21. METABOLIC STEREOSPECIFICITY OF OPIATE AGONIST AND ANTAGONIST DRUGS

Steven H. Pollock and Kenneth Blum

Department of Pharmacology, Division of Drug and Alcohol Abuse, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, 78284.

INTRODUCTION

Pharmacological activity of natural and synthetic opiates is stereospecific with respect to chemical configuration, *i.e.* (-)-enantiomers generally are biologically active, whereas, (+)-enantiomers usually tend to be less active or inactive even as antagonists. Metabolic stereospecificity also occurs and is of interest because it may provide insight into stereoselective mechanisms. Various pathways have been established in metabolism of opiates and some of these routes have been reviewed by Way and Adler (1) and later by Way (2). Recently pathways in metabolism of the narcotic antagonist naloxone (N-allyl-7,8-dihydro-14-hydroxy-normorphinone) have been delimited by Misra *et al*. (3) (Figure 1).

The pathway of N-dealkylation for which enantiomeric substrate stereoselectivity studies have been reported will be considered first for historical perspective, but attention will be focused primarily on 6-keto reduction of naloxone and other dihydromorphinone derivatives for which metabolic product stereoselectivity is operative producing α and β-hydroxy diastereomers. Then other types of opiate metabolic stereospecificity will be considered.

N-DEALKYLATION

N-dealkylation is a major metabolic route for naloxone in the rat (3) and also has been reported as a minor biotransformation pathway for this antagonist in man (4). Naloxone has not been subjected to a metabolic stereoselective comparison with its enantiomorph but studies have been conducted employing structurally related compounds. Initial findings that hepatic microsomal N-demethylating enzymes and opiate analgesic receptors are alike with respect not only to substrates and stereospecificity (5) but also to antagonism by

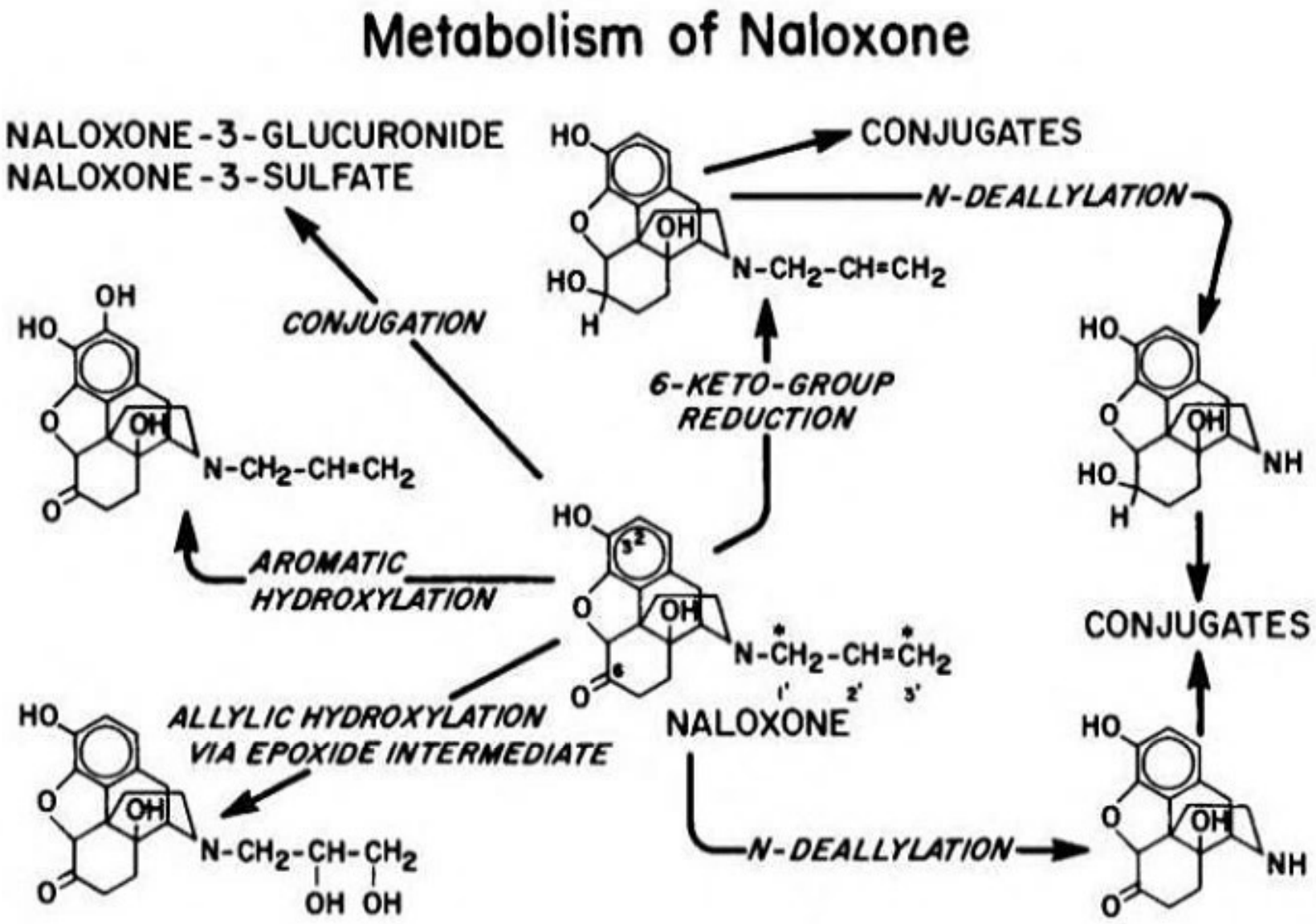


Figure 1

N-allylnormorphine (6) lead to the hypothesis that N-demethylation might serve as a mechanism in the development of tolerance to narcotic drugs (7). *"The repeated administration of morphine* [in vivo] *reduced both enzymatic demethylation and pharmacological response" (7).* Futhermore, *in vivo* administration of N-allylnormorphine not only blocked development of tolerance to morphine but also blocked reduction of N-demethylating activity (7). The tolerance theory proposed by Axelrod was soon doubted by Elison *et al.* (8) who observed on the basis of kinetic parameters (stable Km but decreasing Vmax) that the narcotic N-demethylating system diminished in quantity without a change in enzyme quality. Attention was also focused on this pathway when oxidative N-dealkylation to produce nor-compounds was suggested by Beckett, Casey and Harper (9) *"to be in the reaction sequence leading to analgesia."* Nalorphine (N-allyl-normorphine) was soon reported, however, to be N-dealkylated at an even faster rate than morphine by male rat hepatic microsomes (10) and both nalorphine and morphine were determined to yield normorphine in brain of living rats (11).

Species differences have been detected in the enzymatic demethylation of narcotic drugs. Whereas rat liver enzyme preparations were found to demethylate morphine at a faster rate than observed for rabbit liver preparations, *l*-methadone and meperidine were demethylated faster by rabbit than rat

hepatic preparations suggesting that more than one type of opiate N-dealkylating enzyme may occur (5). Furthermore, a disproportion in N- and O-demethylating capacities of different species with respect to various opiates has been observed and suggests that different enzymes may be responsible for the two types of demethylation (12).

A marked sex difference in opiate N-dealkylating ability has also been observed. Female rat liver enzyme preparations are less active than such preparations from male rat liver in N-demethylating morphine, *l*-methadone, and meperidine and sex hormonal regulation appears to be operative since "*administration of estradiol to male rats results in a decrease in enzyme activity while treatment of female rats with testosterone enhances enzyme activity*" (5).

N-dealkylation substrate stereoselectivity studies of various opiate enantiomers have been reviewed by Jenner and Testa (13) and from their review it indeed becomes readily apparent that there are many conflicting results, the interpretation of which "*is impossible until a clear picture of the stereoselective processes emerges*" (13). Elison *et al.* (8), for instance, found that inactive or less active *dextro* rotatory (+) isomers of various opiates were N-demethylated more slowly than active *levo* rotatory (-) isomers in agreement with findings by Axelrod (5). When Takemori and Mannering (12), however, examined methyl substituted 3-hydroxy-morphinans for N- and O-demethylation by male mouse and rat hepatic preparations, no stereoselectivity for N- and little for O-dealkylation was observed whereas a prominent substrate stereoselectivity for N,O-demethylation occurred (Table 1).

TABLE 1

Demethylation of l- and d-isomers of Morphinan-type Drugs

Type of Demethylation	Compound	μmoles HCHO formed/g hepatic tissue: mouse minutes 15	mouse minutes 120	rat minutes 15	rat minutes 120
N–	*l*-3-hydroxy-N-methylmorphinan (Dromoran)	0.58	0.64	0.28	0.40
N–	*d*-3-hydroxy-N-methylmorphinan (Dextrorphan)	0.50	0.58	0.28	0.32
O–	*l*-3-methoxymorphinan	0.80	0.92	0.68	0.96
O–	*d*-3-methoxymorphinan	0.62	0.86	0.48	0.76
N, O–	*l*-3-methoxy-N-methylmorphinan (Levomethorphan)	2.06	2.60	2.16	2.98
N,O–	*d*-3-methoxy-N-methylmorphinan (Dextromethorphan)	1.46	1.82	1.02	1.64

[Data from Takemori and Mannering (12).]

TABLE 2
Inhibitory Effect of 1- and d-3-hydroxy-N-allyl-morphinan on the Demethylation of 1- and d-isomers of Morphinan-type Drugs by Livers of Mice

Compound	μmoles HCHO found/g tissue per 19 min.	Percent Inhibition
l-3-hydroxy-N-methylmorphinan	0.58	
l-3-hydroxy-N-methylmorphinan + *l*-3-hydroxy-N-allylmorphinan	0.36	38
l-3-hydroxy-N-methylmorphinan + *d*-3-hydroxy-N-allylmorphinan	0.36	38
d-3-hydroxy-N-methylmorphinan	0.54	
d-3-hydroxy-N-methylmorphinan + *l*-3-hydroxy-N-allylmorphinan	0.32	41
d-3-hydroxy-N-methylmorphinan + *d*-3-hydroxy-N-allylmorphinan	0.30	44
l-3-methoxy-N-methylmorphinan	2.50	
l-3-methoxy-N-methylmorphinan + *l*-3-hydroxy-N-allylmorphinan	1.66	34
l-3-methoxy-N-methylmorphinan + *d*-3-hydroxy-N-allymorphinan	1.76	30
d-3-methoxy-N-methylmorphinan	1.72	
d-3-methoxy-N-methylmorphinan + *l*-3-hydroxy-N-allylmorphinan	1.06	38
d-3-methoxy-N-methylmorphinan + *d*-3-hydroxy-N-allylmorphinan	1.06	38

(1 μmole allyl derivative was employed to antagonize 4 μmole morphinan substrate)

[Data from Takemori and Mannering (12).]

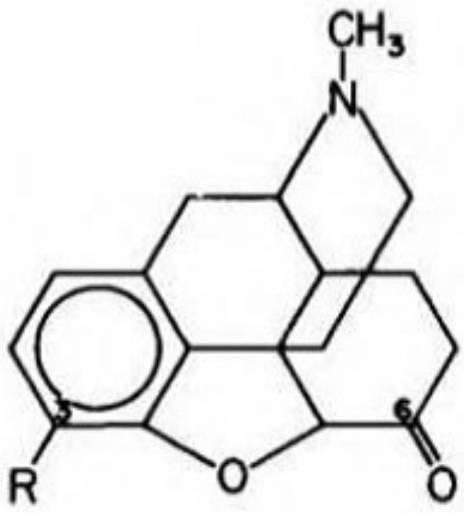
Curiously, inhibition of N-, O-, and N,O-demethylation of methyl substituted 3-hydroxymorphinans by *d*- and *l*-enantiomers of 3-hydroxy-N-allylmorphinan failed to show stereospecificity (Table 2).

The lack of stereospecificity observed in the antagonism of demethylation of methyl substituted 3-hydroxymorphinans by (+)- and (-)-isomers of 3-hydroxy-N-allylmorphinan suggests a dissimilarity between receptors for demethylation and analgesia (12).

6-KETO REDUCTION

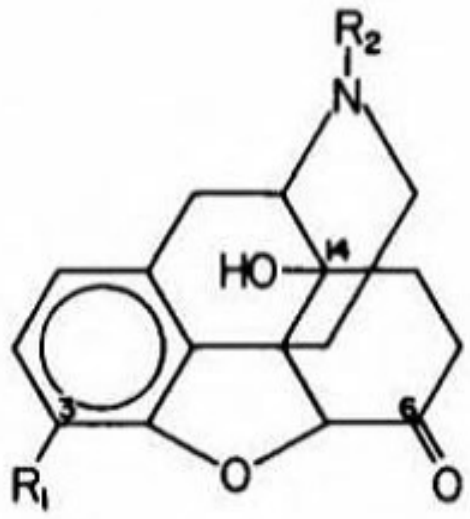
6-Keto reduction had been observed to be an important metabolic pathway for naloxone in chicken hens, the major urinary metabolite identified as N-allyl-14-hydroxydihydro-normorphine (EN-2265) as a 3-glucuronide (14). The principal urinary metabolite of naloxone in male human volunteers and male rabbits was found by Fujimoto (14,15) to be naloxone-3-glucuronide. Weinstein *et al*. (4,16) using a more sensitive thin layer chromatography technique later detected what they believed to be EN-2265 in hydrolyzed human urine and in hydrolyzed glucuronides isolated from rabbit urine, thereby establishing 6-keto reduction as a minor metabolic pathway for naloxone (Narcan^{R}) in mammals. Despite widespread clinical use, metabolic information on structurally related compounds such as the potent analgetics dihydromorphinone (Dilaudid^{R}), oxymorphone (Numorphan^{R}), oxycodone (Percodan^{R}) and the antitussive hydrocodone (Hycodan^{R}, Hycomine^{R}, Hycotuss^{R}) (Figure 2) was not available.

From considerations of the chemical similarity of the double bond in the allyl group of naloxone and the cyclo-propyl group in the N-substitute of naltrexone (17), it was surprising when a compound having a 6-isomorphine configuration was isolated as a major urinary metabolite of naltrexone in man (18) (Figure 3). Attention was thus focused on metabolic product stereoselectivity of opiates. The novel naltrexone metabolite was later confirmed to be N-cyclo-propylmethyl-6-*iso*-7,8-dihydro-14-hydroxynormorphine (19) and soon this stereoselected metabolite (6β-naltrexol) was identified as an *in vivo* reduction product in guinea pig (20) and an *in vitro* metabolite produced by an hepatic preparation from male rabbit (21). The rabbit preparation similarly yielded 6β-naloxol from naloxone (21). GLC analysis indicated that 97% of the guinea pig naltrexone reduction product was 6β-naltrexol and 3% 6α-naltrexol (20), but the formation of 6α-naltrexol and 6α-naloxol by rabbit preparations was doubted on the basis of Fourier transform computerized NMR spectroscopy (21).



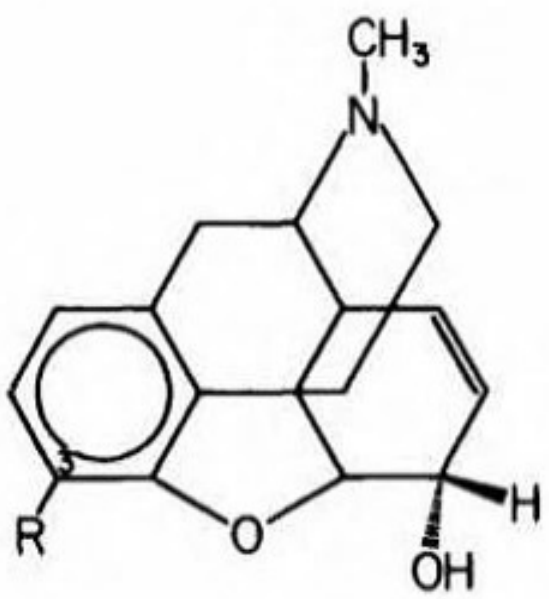
R=OH
Hydromorphone
(7,8-dihydromorphinone)

R=OCH3
Hydrocodone
(7,8-dihydrocodeinone)



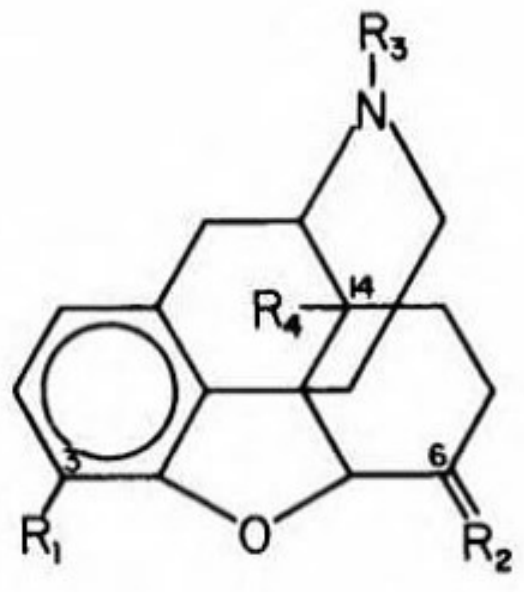
R1=OH	R2=
Oxymorphone	CH_3
(14-OH-7,8-dihydromorphinone)	
Naloxone	CH_2-CH=CH_2
Naltrexone	CH_2-CH<CH_2 CH_2
EN-1620	CH_2-CH=C<CH_2 CH_2
EN-1655	CH_2-◇
R1=OCH_3	R2=
Oxycodone	CH_3

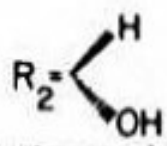
Fig. 2. Some dihydromorphinones and dihydrocodeinones



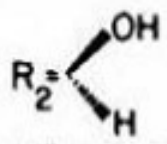
R=OH
Morphine

R=OCH_3
Codeine





Dihydromorphine and Dihydrocodeine Derivatives 6-Alpha-OH



Dihydroisomorphine and Dihydroisocodeine Derivatives 6-Beta-OH

Fig. 3. Stereoconfiguration at C-6 in the dihydromorphine and dihydroisomorphine series.

DIHYDROMORPHINONE KETONE REDUCTASES

Prior to stereochemical studies of naloxone and naltrexone-6-ketone reduction, investigation was launched to characterize the enzymes responsible for naloxone reduction in chicken and rabbit. Pollock (22,23) found the greatest reduction to occur by NADPH dependent enzymes from hepatic cytosol and developed a sensitive radioassay. The standard assay mixture consisted of 7.9 µmole glucose-6-phosphate, 0.25 µmole $NADP^+$, 2 units crystalline glucose-6-phosphate dehydrogenase, purified reductase, 1 µmole naloxone as substrate, and 0.01 µmole ^{14}C-naloxone as a tracer in a final volume of 1.0 ml 0.05M $KH_2PO_4/NaOH$ pH 7.4 buffer. Analysis was performed by scintillation counting of excised TLC spots located with iodoplatinate spray reagent.

Pollock (22,23) was able to distinguish these enzymes from other keto reductases such as aromatic alpha-keto acid reductase (24), lactic dehydrogenase (25), liver alcohol dehydrogenase (26,27,28,29), the beta-ketoacyl-ACP reductase component of fatty acid synthetase (30,31,32), an enzyme designated AK-reductase (33), and an alpha, beta-unsaturated ketone reductase (34). Since dihydromorphinone and its derivatives naltrexone and EN-1655 (N-cyclobutylmethyl-14-hydroxynormorphinone) were also observed by TLC to undergo 6-keto reduction *in vitro*, the novel reductases from hepatic cytosol were designated dihydromorphinone ketone reductases (DMKR's) (22,23).

Cofactor regulation of DMKR's appears to be operative since $NADP^+$ was found to be inhibitory in both rabbit (Table 3) and chicken DMKR preparations (22). Lower activity is observed when NADPH is employed without an NADPH generating system. This phenomenon evidently results from accumulation of $NADP^+$.

For chicken liver peak DMKR activity occurred in fractions salted-out between 50% and 65% ammonium sulfate saturation and for rabbit it occurred in fractions salted-out between 50% and 60% ammonium sulfate saturation. Prior to dialysis of ammonium sulfate salted-out fractions, DMKR activity was found to vary with protein concentration. A ceiling effect was observed with both the 100,000 x g supernatant and undialyzed salted-out fractions of chicken and rabbit liver (Figure 4).

Prior to the dialysis specific DMKR activity (nanomoles naloxone reduced/mg protein/min) was found to decrease as protein concentration was increased and incubation time was prolonged (Figure 5).

These findings suggest that the concentration of an inhibitor is increased along with increases of hepatic protein in the assay mixture. After dialysis of peak

TABLE 3

Inhibitory Effect of $NADP^+$

Group		% Naloxone Reduction Mean	SEM	N
1	0.25 μmole $NADP^+$ with standard generating system	27.700	0.90	2
2	1.0 μmole NADPH	21.333	0.64	6
3	1.0 μmole NADPH plus 0.25 μmole $NADP^+$	18.700	0.47	3

ANOVA F Ratio = 26.26 Treatment df = 2 Error df = 10 $P < .001$

Student Newman Keuls Test

Mean		Mean	Difference	LSR	$P < 0.05$
27.700	-	21.333	6.367	2.247	significant
21.333	-	18.700	2.633	2.595	significant
27.700	-	18.700	9.000	3.595	significant

Dialyzed 50%-60% ammonium sulfate salted-out fraction of rabbit 100,000 x g hepatic supernatant was employed to reduce 10^{-3} M naloxone in 10 minute assays employing three different cofactor conditions. Statistical analysis was by analysis of variance and a Student Newman Keuls Test (35).

ammonium sulfate salted-out fractions specific activity was found to be independent of protein concentration for naloxone reduction up to 30% and 60% respectively for rabbit and chicken preparations in standard assays of ten minutes. Furthermore, the Michaelis-Menten constant (Km) of naloxone

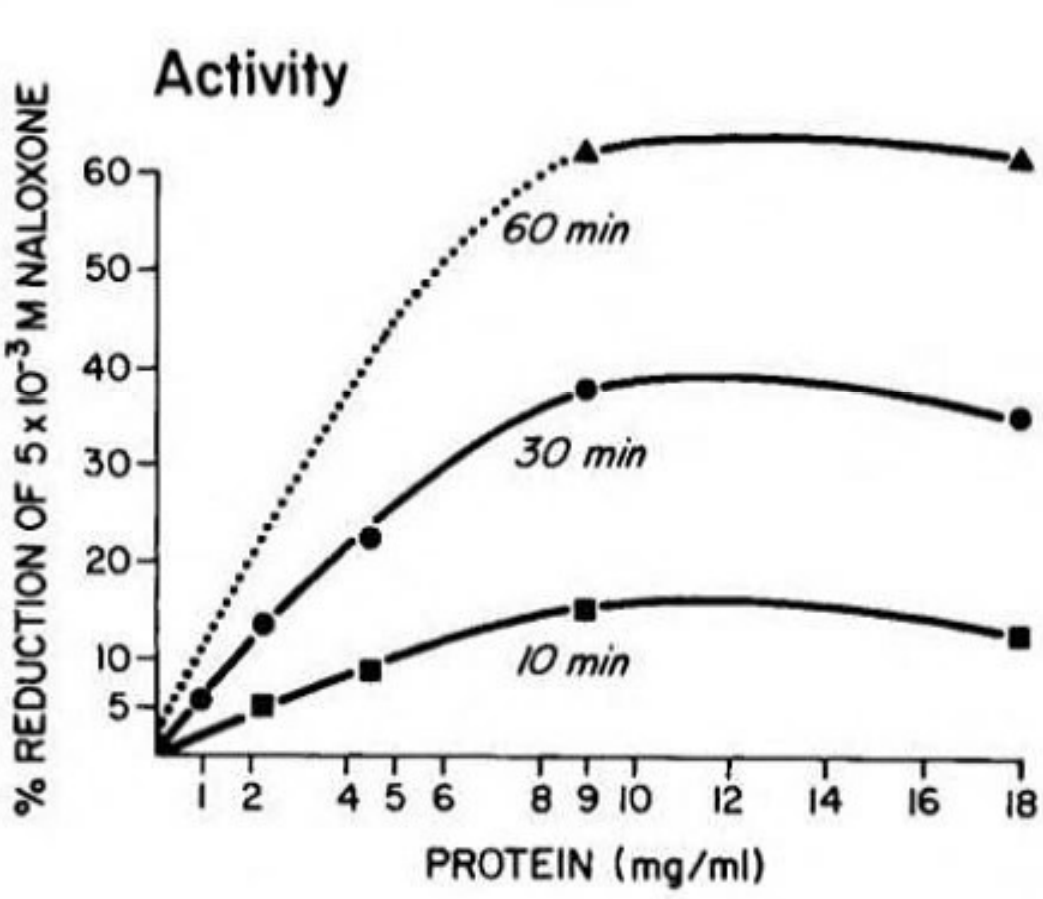


Fig. 4. Assays were performed using rabbit hepatic enzyme aliquots salted-out between 50% and 60% ammonium sulfate saturation.

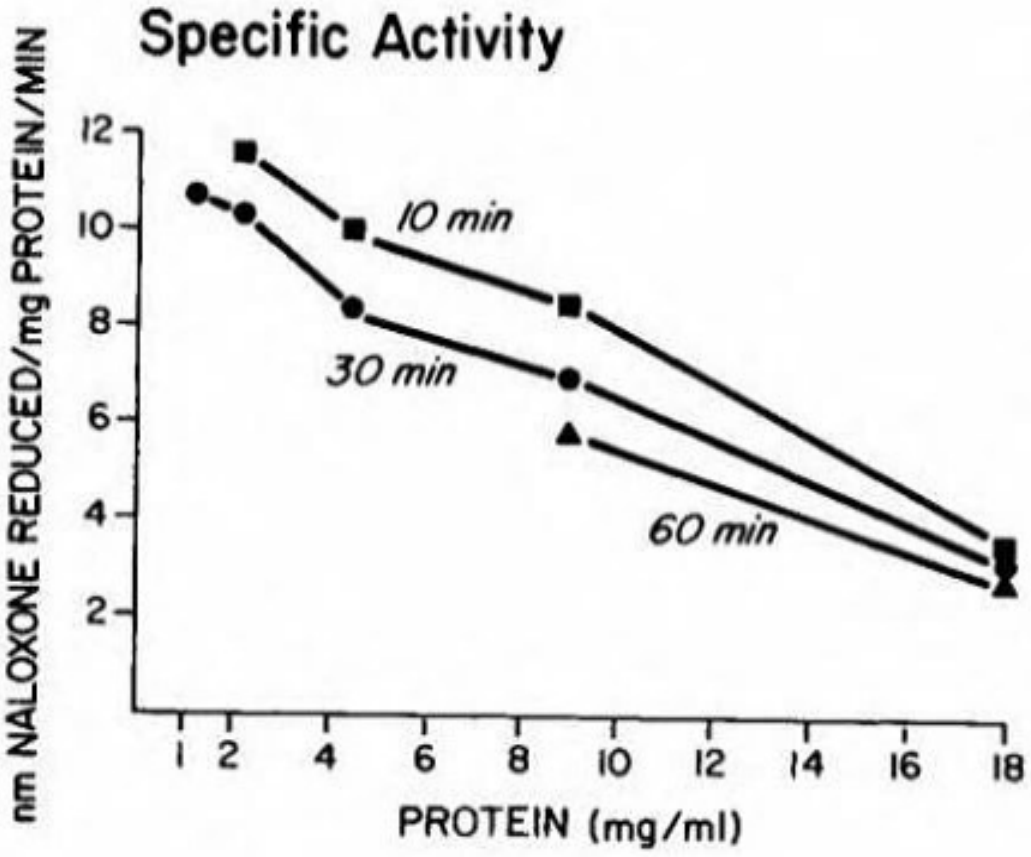


Fig. 5. Specific activities were determined using the same rabbit hepatic assays for which data were reported in Figure 4.

toward rabbit hepatic DMKR was measured at 10^{-3}M using the 100,000 x g supernatant but was found to be in the range of 10^{-4}M (once even measured at 9.0 x 10^{-5}M) after dialysis of peak ammonium sulfate fractions. Km findings for the chicken enzyme were similar, but specific activities of dialyzed peak ammonium sulfate salted-out fractions in standard assays tend to be in the range of 10-12 units for rabbit and 20 units for chicken hepatic DMKR's. These specific activities were not found to increase with increases in substrate concentration and thus represent measured Vmax's.

The pH optima for rabbit and chicken DMKR's were found to be approximately 7.4 and 7.0 respectively and the pH activity curves have a similar paraboloid shape. Molecular weights of rabbit and chicken DMKR's were determined to be in the range of 50,000 and 60,000 daltons respectively as estimated by G-100 Sephadex column chromatography and peak specific activities for eluates of nondialyzed salted-out enzyme fractions were in the range of 25 units for both rabbit and chicken preparations.

A direct comparison of reduction velocities of naloxone and naltrexone at 10^{-3}M by rabbit and chicken preparations indicated no significant difference and reduction velocities were similar for the two substrates at concentrations near 10^{-4}M using rabbit Sephadex enzyme eluate. Reduction velocities of naloxone and naltrexone were also similar when compared indirectly by using 10^{-5}M ^{14}C-naloxone as tracer for each. Although for rabbit DMKR preparation hydromorphone behaved similarly to naloxone by indirect assay, EN-1655 is significantly different (Table 4). Hydromorphone and EN-1655 were found to be similar to naloxone by indirect assay with chicken DMKR preparation.

TABLE 4

Indirect Comparison of Naloxone, Hydromorphone and EN-1655 as Substrates

10^{-3} M Substrate	% Reduction of 10^{-5} M ^{14}C-Naloxone			
Exp. 1	*Mean*	*SEM*	*N*	*P*
Naloxone	21.0	1.14	4	
Hydromorphone	20.2	.42	4	N.S.
Exp. 2				
Naloxone	17.8	.27	4	
EN-1655	10.3	.32	5	< .001

Experiments 1 and 2 were done on different days using dialyzed peak ammonium sulfate fractions of rabbit DMKR. Statistical analysis was done by a 2-tailed t test.

Rabbit and chicken DMKR preparations were found to be different in their sensitivities to inhibitors relative to the isotope dilution effect of nonlabelled naloxone (Table 5).

The dissociative anesthetic ketamine (2-chlorophenyl-2 methylaminocyclohexanone) is a stronger inhibitor of chicken DMKR than of the rabbit DMKR system, whereas morphine is more inhibitory toward the rabbit enzyme system. Similarly dihydromorphine, nalbuphine (6α-hydroxy reduction product of EN-1655), EN-2265 (6α-naloxol), and EN-2260 (6α-naltrexol) demonstrated very little product inhibition toward chicken DMKR but in comparison are strongly inhibitory toward the rabbit enzyme system (22,23).

After DMKR enzyme characterization studies were completed by Pollock (22,23), Roerig *et al*. (36) working in the same laboratory undertook a follow-up investigation of the same enzyme systems. Unfortunately, those investigators failed to account for impurities present in the ^{14}C-naloxone and ^{3}H-naltrexone which chromatographed as ca. 3% naloxol and 1% naltrexol respectively. The reliability of their enzyme activity and Km calculations is thus in serious doubt, expecially when low velocities are involved. Not only did Roerig *et al*. employ nondialyzed ammonium sulfate salted-out protein fractions for which specific activities may have varied with their protein concentrations but they also failed to account for the substantial change in substrate concentrations which occurred during their thirty minute assays for Km calculations. It is thus no wonder that their Km values for naloxone were found to be almost an order to magnitude above those obtained by Pollock (22,23) for dialyzed enzyme preparations. While the kinetic data of Roerig *et al*. (36) are entirely unreliable, their inhibitor

TABLE 5

Relative Inhibitory Effect of Various Compounds

Inhibitor	Relative Inhibitory Effect at 10^{-3} M	
	Rabbit	Chicken
Ketamine	0.8	1.2
Morphine	0.9	< 0.1
Hydromorphine	1.0	0.2
Nalbuphine	1.0	0.1
EN-2265	0.8	0.3
EN-2260	0.7	0.3
Naloxone-3-glucuronide	0.5	0.3
EN-2265-3-glucuronide	0.4	0.4
D-glucuronic acid	0.1	0.2
L-tyrosine	0.1	0.4

The relative inhibitory effect at 10^{-3} M was estimated by the following calculations:
Where, x = % reduction of 10^{-5} M radionaloxone alone
y = % reduction of 10^{-5} M radionaloxone with 10^{-3} M inhibitor present
z = % reduction of 10^{-5} M radionaloxone with 10^{-3} M nonlabeled naloxone
% inhibition by inhibitor = 100 (x-y)/x
% inhibition by isotope dilution effect = 100 (x-z)/x
Therefore, relative inhibitory effect of inhibitor = (x-y)/(x-z)

[Data from Pollock (23)]

findings nevertheless are confirmatory and supplement results obtained by Pollock (22,23).

SOME STEREOSPECIFIC STUDIES OF DMKR ENZYME SYSTEMS

Assays of hepatic cytosol preparations had indicated a widespread species distribution of DMKR activity (22,23) and stereospecific studies of this activity were undertaken by Pollock and Dear (37) employing hydromorphone, naloxone and naltrexone as substrates. Additional substrates were reduced with rabbit and chicken DMKR preparations and evaluated by Pollock (38).

Naloxone·HCl, EN-2265 (6α-naloxol), naltrexone·HCl, EN-2260A (6α-naltrexol·HCl), Oxymorphone·HCl, EN-2370 (14-hydroxydihydromorphine), nalmexone·HCl (EN-1620A), EN-2261K (6α-nalmexol), EN-1655A, nalbuphine·HCl, hydrocodone bitartrate, and oxycodone·HCl were provided gratis from Endo Laboratories, Inc. Hydromorphone·HCl was obtained from Knoll Pharmaceutical Company. Dihydromorphine, dihydrocodeine, 6β-naltrexol·HCl, and an additional sample of 6α-naltrexol·HCl were supplied by the National Institute on Drug Abuse.

Methodology, findings, and observations follow.

Enzyme Preparation

Fresh livers were obtained from animals sacrificed by decapitation except for calf and swine liver which was purchased from a local supermarket. Hepatic tissue was homogenized in a Waring blender using 0.106 M K_2HPO_4/0.07 M $KHCO_3$ pH 7.9 buffer (2 ml/gram tissue). The crude homogenate was centrifuged for thirty minutes at 15,000 x g in a Sorvall Centrifuge. The supernatant was then centrifuged for ninety minutes in a Beckman Ultracentrifuge (Model L2-65) at R_{av} = 95,100 x g (R_{max} = 131,000 x g). After removal of most of the surface lipid layer with a Kimwipes^R tissue, the aqueous supernatant was transferred via Dispo-pipettes into a glass beaker. Swarz-Mann ultra pure special enzyme grade ammonium sulfate was used for enzyme salting-out, the quantities employed having been calculated from an equation based on saturation at 0°C (39). Fractions were collected by centrifugation at 15,000 x g for fifteen minutes. The desired fractions were resuspended in a minimal volume of dialysis buffer and dialyzed against one liter 0.005 M KH_2PO_4/NaOH buffer (pH 7.0) for twenty-four hours. All buffers employed in enzyme preparations were chilled to 4°C and an effort was made to keep tissue and enzyme preparations cold by working with an ice water bath or in a 4°C coldroom.

Enzyme Assay

6-Carbinols of dihydromorphinones and dihydrocodeinones were produced in 250 ml Ehrlenmeyer flasks by incubation of 6.0×10^{-5} mole substrate with 240 µmole glucose-6-phosphate (from Sigma), 7.5 µmole $NADP^+$ (from Sigma), 60 units crystalline glucose-6-phosphate dehydrogenase (Sigma type XV from Baker's yeast), and 3 ml dialyzed ammonium sulfate salted-out enzyme fraction in a final volume of 30 ml 0.05 M KH_2PO_4/NaOH buffer (pH 7.4) in a Dubnoff Metabolic Incubator at 60 oscillations/minute for two hours at 37°C. The assay mixtures were then decanted into glass stoppered bottles containing 30 ml 1.0 M $NaHCO_3/Na_2CO_3$ (pH 10.0) buffer and 60 ml ethyl acetate. After several minutes of vigorous shaking to extract metabolites into the organic phase, the phases were separated by centrifugation at 9,000 x g for five minutes. The ethyl acetate phase (top layer) was transferred by Dispo-pipette to a boiling flask and evaporated at 37°C under nitrogen.

Boiling flask residue containing metabolites and unutilized substrate was dissolved in 0.5 ml pyridine, 10 µl was transferred to an acylation tube, and silylation was accomplished with addition of 10 µl N,O-*bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Analabs. The reaction was allowed to proceed for fifteen minutes. The silylation

mixture was evaporated under dried N_2 and the trimethylsilyl derivatives resuspended in 30 µl ethyl acetate. Samples of 1.5 µl were analyzed by gas-liquid chromatography employing a glass column (l = 2m, i.d. = 0.25mm, liquid phase = 3% OV-17 on GasChrom Q with 100/120 mesh) in a Varian GC (Series 1700) with a flame-ionization detector. Dried nitrogen (30 ml/min) was used as the carrier gas and hydrogen and air flows were 25 and 300 ml/min respectively. The column temperature was 255°C and the injector and detector temperatures were 265° and 275° respectively.

GLC-MS was obtained by integration of a Finnigan 1015 S/L Mass Spectrometer (Model 1015C) and a Varian GC (Series 1400). A Systems Industries System/150 data output device was employed to provide data printout. By GLC-MS the identity of derivatized standards and metabolites was established. Quantitation was based on peak height measurement. When 6-keto reduction was complete, metabolites were silylated to *bis*-TMS derivatives for this purpose. Since when reduction was incomplete a mixture of *bis* and *mono*-TMS derivatized metabolites was usually obtained, both derivatives were accounted for in quantitation.

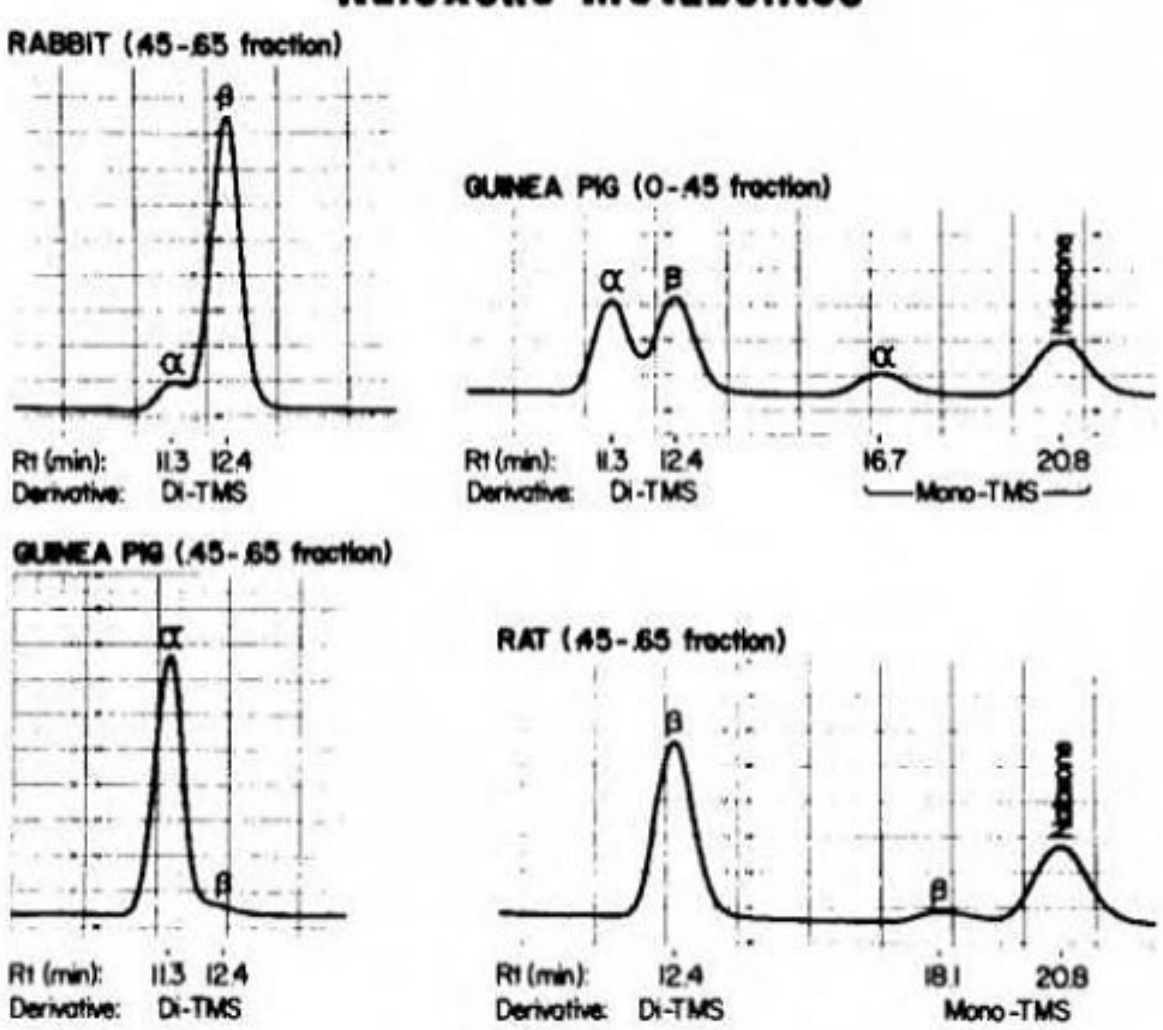
Findings

Bis- and *mono*-silylated derivatives of reduced dihydromorphinones having the 6α-hydroxy configuration had shorter retention times than those having the 6β-hydroxy configuration (Figures 6,7). Trisilylated 6β-naloxol and 6β-naltrexol were ascertained to have shorter retention times than trisilylated 6α-naloxol and 6α-naltrexol respectively.

The 6-ketone moiety of naloxone was substantially reduced *in vitro* by hepatic preparations from most species studied (Table 6).

The 45-70% ammonium sulfate salted-out hepatic DMKR fraction of pigeon (White King, 1 M and 1F) and chicken (White Leghorn, 2 M and 2 F) produced only 6α-naloxol as did a 0-45% fraction of DMKR from chicken (2 M). A 45-70% DMKR fraction from seven immature chickens (age between one and two weeks) resulted in complete naloxone reduction to 6α-naloxol. Muscovy duck (1 M) preparation produced both 6α and 6β-hydroxy diastereomers. Only the 6β-hydroxy epimer was detected after naloxone reduction by rat (Sprague-Dawley, 2 M) and swine 45-65% ammonium sulfate salted-out DMKR preparations, but the same fraction of calf, rhesus monkey (1 M), and New Zealand White Rabbit DMKR's produced some 6α-naloxol in addition to 6β-naloxol. The ratio of 6α-naloxol to 6β-naloxol was about 1:8 whether produced by the 45-60% fraction from male or female rabbit. The 0-45% fraction from the same rabbit sources yielded a similar predominance of 6β-naloxol. The 45-65%

fraction from two immature rabbits (age ten and thirteen weeks) was also active, completely reducing naloxone to 19% 6α-naloxol and 81% 6β-naloxol. Furthermore, a 45-65% fraction



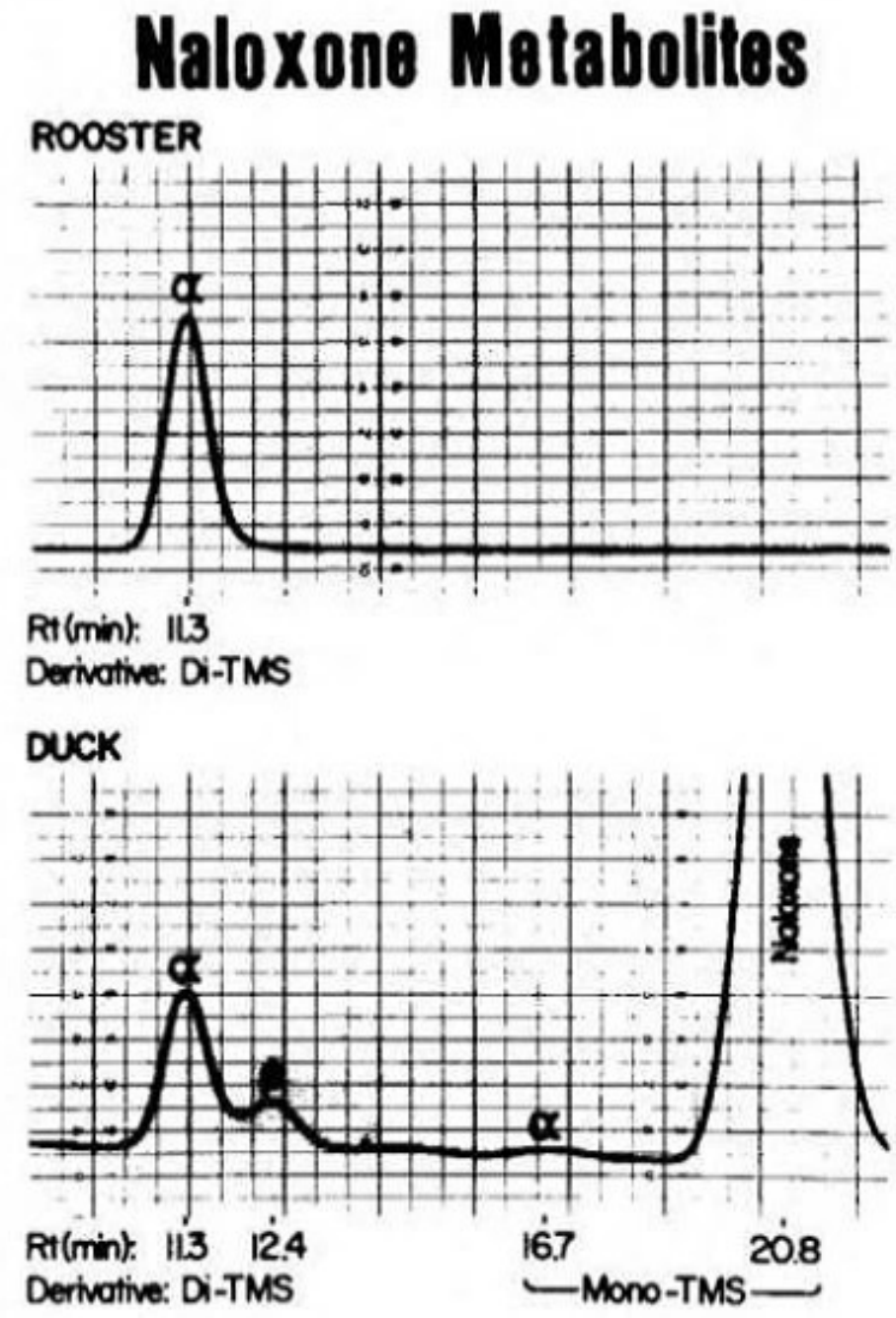


Fig. 6. Display chromatograms at original chart speed of 2cm/min.

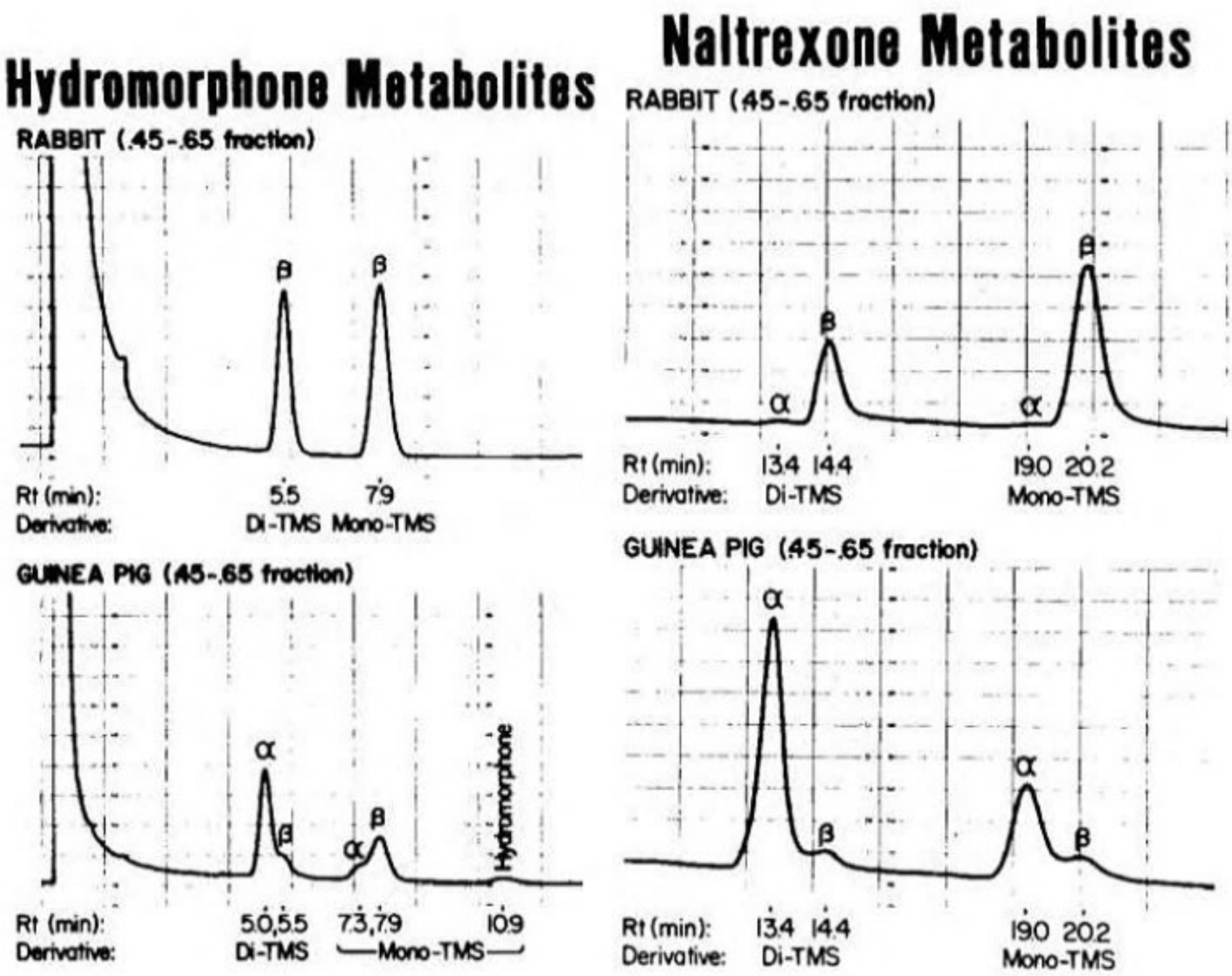


Fig. 7. Display chromatograms at original chart speed of 2cm/min.

from six neonate rabbits (less than one day old) completely reduced naloxone to about 5% of the 6α-hydroxy epimer and 95% of the 6β-hydroxy epimer. The 45-65% fraction of guinea pig (Harther strain, 3 M) DMKR's completely reduced naloxone to predominantly the 6α-hydroxy epimer (95%) whereas the 0-45% fraction from the same guinea pig source reduced naloxone 77% to almost an equal quantity of 6α and 6β-hydroxy diastereomers (α:β = 49:51). The 45-65% fractions of two cat livers assayed independently were without DMKR activity. A 0-45% fraction of one feline liver was assayed and yielded about 1% naloxone reduction to a metabolite which chromatographed as 6α-naloxol.

Hydromorphone was reduced by chicken (2 M) DMKR and pigeon (1 M and 1 F) to dihydromorphine (6α-hydroxy), whereas it was reduced by muscovy duck (1 M) DMKR preparation to 6α and 6β-hydroxy diastereomers. A 0-45% fraction of feline hepatic preparation was inactive toward hydromorphone but reduced about 5% naltrexone to a metabolite which chromatographed as 6α-naltrexol. A 45-65% fraction of cat liver was ineffective in reducing either substrate. Hydro-

TABLE 6

Species Stereospecificity Survey with Naloxone

Order	Genus and species Common name (number)	Configuration %α	Configuration %β	Naloxone Reduction %
Anseriformes	*Cairina moschata* muscovy duck (1)	76	24	26
Columbiformes	*Columba livia* pigeons (2)	100	0	12
Galliformes	*Gallus gallus* chickens (4)	100	0	100
Artiodactyla	*Bos taurus* calf (1)	5	95	56
	Sus scrofa swine (1)	0	100	38
Carnivora	*Felis catus* cat (2)	--	--	0
Lagomorpha	*Oryctolagus cuniculus* rabbit (4)	11	89	100
Primata	*Macaca rhesus* rhesus monkey (1)	14	86	41
Rodentia	*Cavia porcellus* guinea pigs (3)	95	5	100
	Rattus norvegicus white rats (2)	0	100	76

The 45-70% $(NH_4)_2SO_4$ salted-out DMKR fraction of avian livers and the 45-65% fraction of mammalian livers were employed to reduce naloxone. The chicken and rabbit liver assays were performed twice, once with preparations from two males and once with preparations from two females. The two cat livers were assayed independently. Other assays were performed once.

morphone and naltrexone were reduced by guinea pig DMKR equivalent amount of 6α-hydroxy and 6β-hydroxy diastereomers produced by the 0-45% fraction and a marked predominance of the 6α-hydroxy epimer formed by the 45-65% fraction. In contrast, a 45-65% fraction of DMKR's from male rabbit completely reduced hydromorphone to only the 6β-hydroxy carbinol, *i.e.* dihydroisomorphine.

A stereospecific comparison of hydromorphone, naloxone, and naltrexone reduction using a DMKR preparation from female rabbit revealed a significant difference between the three substrates (Table 7).

In a separate assay using the DMKR preparation from female rabbit liver, oxymorphone was completely reduced to yield approximately 5% 14-hydroxydihydromorphine (6α-hydroxy) and 95% 14-hydroxydihydroisomorphine (6β-hydroxy). Using a 45-65% fraction of DMKR's prepared from two male rabbit livers, additional substrates were simultaneously reduced. Nalmexone (EN-1620) was reduced to 6-carbinols, 10% of which represented the 6α-hydroxy epimer and 90% of which represented the 6β-hydroxy epimer. EN-1655 was completely reduced to only the 6β-hydroxy carbinol (isonalbuphine). Oxymorphone was completely reduced with this preparation to about 5% of the 6α-hydroxy epimer and 95% of the 6β-hydroxy epimer. Hydrocodone, in

TABLE 7

A Stereospecific Comparison of Substrates

	Hydromorphone	Naltrexone	Naloxone
% α - hydroxy product formed	0, 0, 0	1.8, 0, 2.4	9.7, 9.4, 13.5
Mean	0.00**	1.40*	10.87**
Variance	0.00	1.56	5.22
Standard Deviation	0.00	0.72	1.32
Number	3	3	3

ANOVA F Ratio = 46.36 Treatment df = 2 Error df = 6 $p < .001$

* $p < .01$ (planned comparison ψ)
** $p < .001$ (planned comparison ψ)

The three substrates were each completely reduced in triplicate in simultaneous assays using a dialyzed 45-65% ammonium sulfate salted-out fraction of DMKR's prepared from two female rabbit livers. Statistical analysis was by analysis of variance and planned comparison (40).

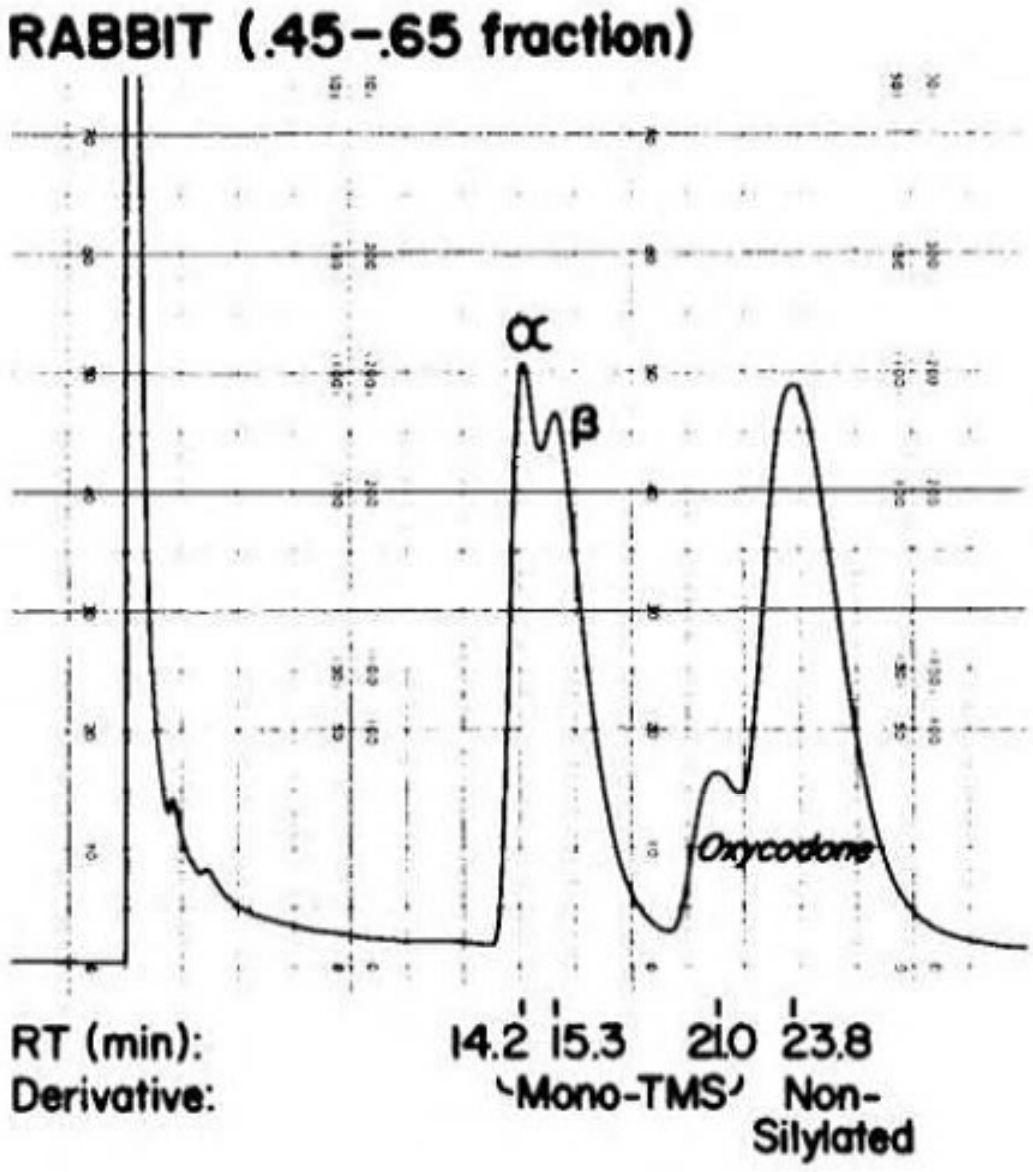


Fig. 8. Chart speed originally at 1/2 cm/min.

contrast, was approximately 29% reduced to only dihydrocodeine (6α-hydroxy), whereas oxycodone was about 60% reduced to 6-carbinols, 52% of which was 14-hydroxydihydrocodeine (6α-hydroxy) and 48% of which was 14-hydroxydihydroisocodeine (6β-hydroxy) (Figure 8). Both hydrocodone and oxycodone were completely reduced with a 45-70% ammonium sulfate salted-out fraction from two rooster livers to 6α-hydroxy carbinols.

No 6-carbinols were detected upon GLC analysis of standard substrates. Standard EN-2265 (6α-naloxol), EN-2260 (6α-naltrexol), 6β-naltrexol, dihydromorphine. and dihydrocodeine were pure according to GLC. A batch of 6α-naltrexol from the National Institute on Drug Abuse contained about 6% 6β-naltrexol, EN-2261 K (6α-nalmexol) contained about 8% 6β-nalmexol, and nalbuphine contained about 4% isonalbuphine. EN-2370 (14-hydroxydihydromorphine) contained about 12% of the 6β-hydroxy epimer.

Mass spectra of the trimethylsilyl derivatives contained a base peak at m/e 73 corresponding to a TMS fragment. When limited mass spectra were obtained for m/e greater than 100, *bis*-TMS-dihydromorphine and the metabolite identified as dihydroisomorphine displayed the same parent and base peak at m/e 431 (Figure 9). The limited mass spectra of *bis*-TMS derivatives of 14-hydroxydihydromorphine and N-substituted analogs such as 6α-naloxol contained a base peak at M^+-15

corresponding to loss of a methyl group from the parent peak, whereas for *bis*-TMS derivatives of 14-hydroxydihydroisomorphine and analogs such as 6β-naloxol the base and parent peaks coincided.

Observations

Mixtures of C-6 epimers containing about 10-15% of the 6β-hydroxy diastereomers have been detected after sodium borohydride reduction of naloxone and naltrexone (41,42), whereas 6-keto reduction of these 14β-hydroxy dihydronormorphinone derivatives with lithium tri-*sec*-butylborohydride has been found to be stereospecific for the 6α-hydroxy epimers (42). In contrast, 6-keto reduction of naloxone and naltrexone with formamidinesulfinic acid has been shown to be stereospecific for the 6β-hydroxy reduction products (43). On the basis of the stereospecificity observed in the enzymatic 6-keto reduction of dihydromorphinones and dihydrocodeinones, one could propose that a limited number of enzymes have a multiplicity of stereospecificities toward these substrates. It is generally regarded in enzymology, however, that different stereospecific activities are the result of catalysis by different stereospecific enzymes (44,45,46,47). This concept is particularly evident with respect to enzymes involved in biotransformation of steroids (48,49). Pollock (38) thus recognized that there are a minimum of at least five different types of enzymes which reduce the 6-ketone moiety of dihydromorphinones and dihydrocodeinones.

DMKR of chicken (Type I DMKR) produces 6α-hydroxy reduction products from dihydromorphinone and 14-hydroxydihydromorphinones whereas a DMKR of rabbit (Type II DMKR) produces 6β-hydroxy metabolites from these substrates. These may be regarded as hydromorphone keto reductases. Rabbit also possesses an oxymorphone keto reductase (Type III DMKR) which produces 6α-hydroxy metabolites from 14-hydroxydihydromorphinones. Although pigeon possesses a Type I DMKR, muscovy duck has enzymes with Type I and Type II DMKR activity. Mammalian DMKR's are predominantly of Type II and to a lesser extent probably of Type III. Guinea pig, however, displays mostly Type I DMKR activity *in vitro* yielding a predominance of 6α-hydroxy carbinols.

Although chicken hens have been shown to reduce naloxone *in vivo* to 6α-naloxol (19), guinea pigs reduce naltrexone *in vivo* almost exclusively (at least 97%) to 6β-naltrexol (10,42). Thus it appears that in the guinea pig, Type II DMKR is the most important *in vivo*. The domestic cat is anomalous not only in that it conjugates naloxone principally with sulfuric acid rather than glucuronic acid (50) but also in that, for all practical purposes, it lacks DMKR activity.

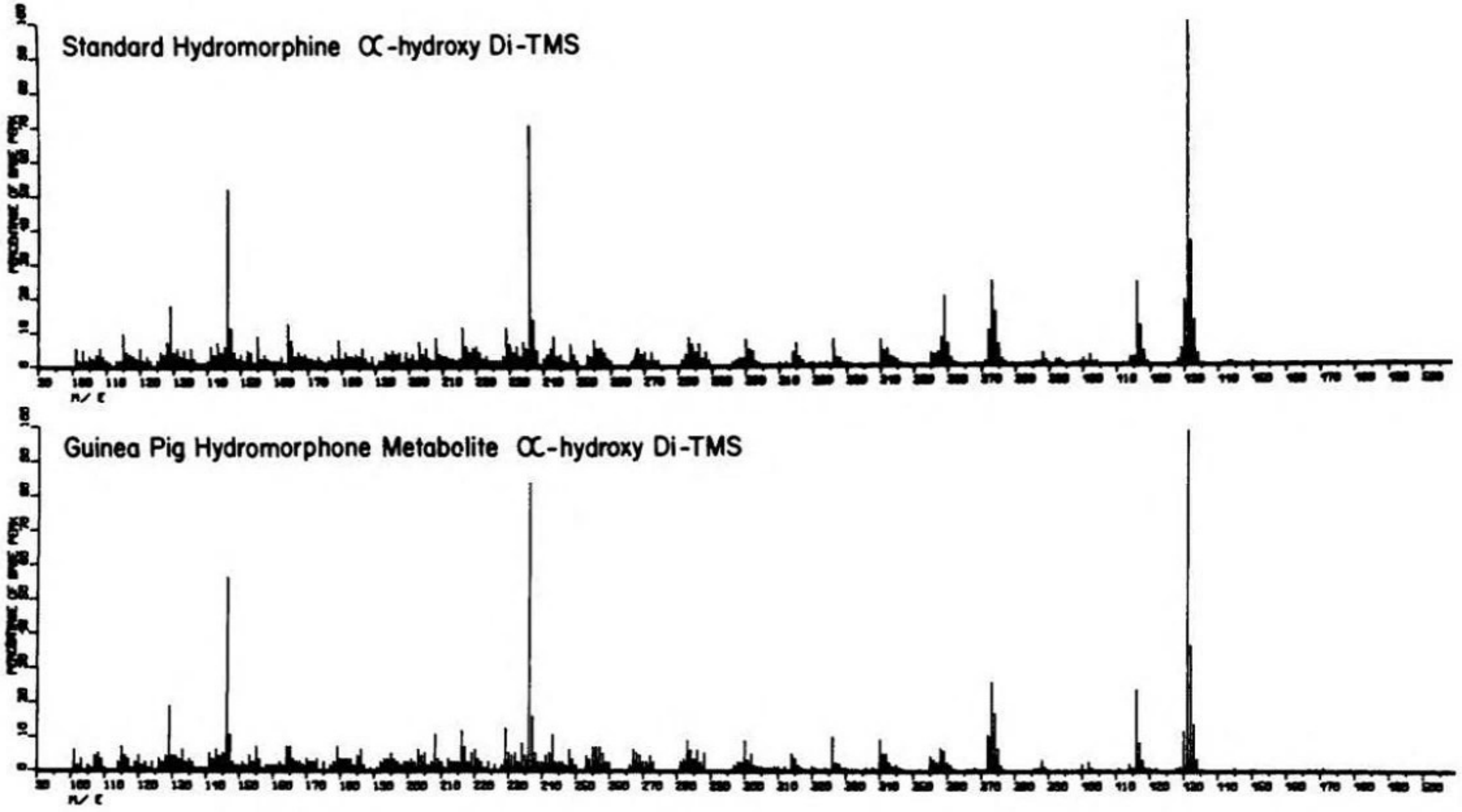


Fig. 9. Limited Mass Spectra of Dihydromorphine and Dihydroisomorphine

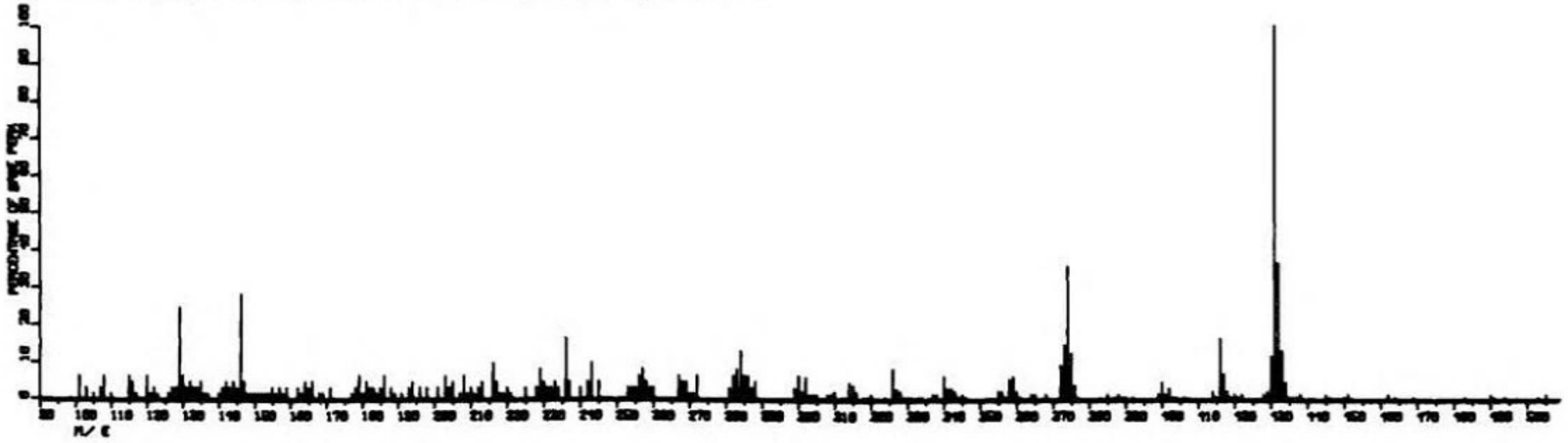


Fig. 9. Limited Mass Spectra of Dihydromorphine and Dihydroisomorphine (continued)

Since with rooster hepatic preparation hydrocodone and and oxycodone are reduced to 6α-hydroxy carbinols as is hydromorphone, the dihydrocodeinone ketone reductase (DCKR) activity in that species may be due to Type I DMKR. Since with rabbit hepatic preparations, however, hydromorphone is reduced to the 6β-hydroxy carbinol whereas hydrocodone is reduced to the 6α-hydroxy carbinol, rabbit hydrocodone keto reductase (Type I DCKR) is a separate enzyme. The reduction of oxycodone by rabbit hepatic preparation at about twice the rate for hydrocodone and to roughly equal proportions of 6α and 6β-hydroxy diastereomers indicates there is also a rabbit oxycodone keto reductase (Type II DCKR). Whereas hydrocodone keto reductase reduces hydrocodone and oxycodone to 6α-hydroxy carbinols, oxycodone keto reductase reduces oxycodone but not hydrocodone to a 6β-hydroxy carbinol.

Since metabolic reactions are theoretically reversible, dihydromorphinone and dihydrocodeinone ketone reductases should be designated as oxido-reductases. Type I DMKR, of which the chicken hepatic enzyme is the prototype, is dihydromorphine: $NADP^+$ oxidoreducatse and Type II DMKR, of which the rabbit hepatic enzyme is the prototype, is dihydroisomorphine: $NADP^+$ oxidoreductase (22,23). Type III DMKR, of which the rabbit hepatic enzyme may be considered the prototype, is a 14-hydroxydihydromorphine: $NADP^+$ oxidoreductase. Type I DCKR (Type IV DMKR), of which the rabbit hepatic enzyme may be considered the prototype, is a dihydrocodeine: $NADP^+$ oxidoreductase. Type II DCKR (Type V DMKR), of which the rabbit hepatic enzyme is also the prototype, is a 14-hydroxydihydroisocodeine: $NADP^+$ oxidoreductase. An alternative designation for Type I DCKR is 3-0-methyldihydromorphine: $NADP^+$ oxidoreductase, whereas Type II DCKR may be regarded as a 3-0-methyl-14-hydroxydihydroisomorphine: $NADP^+$ oxidoreductase.

Although naloxone, naltrexone, and hydromorphone were found to be reduced at similar rates by rabbit hepatic DMKR preparation using a sensitive radioassay (22,23), hydromorphone and naltrexone have now been found to yield significantly more of the 6β-hydroxy reduction product *in vitro* than does naloxone, whereas naloxone yields significantly more of the 6α-hydroxy reduction product. These findings suggest differences in affinity of these compounds toward Type II and Type III DMKR's from rabbit. Since only isonalbuphine (6β-hydroxy diastereomer of nalbuphine) has been detected after reduction of EN-1655 and this substrate appeared to have significantly greater affinity for rabbit hepatic Type II DMKR by indirect radioassay (22,23), EN-1655 seems to be the best substrate tested for rabbit Type II DMKR. Oxymorphone seems to be intermediate between naltrexone and naloxone in the α:β ratio of 6-carbinols produced by rabbit DMKR's. Thus the relative

order of affinity of these dihydromorphinones toward the prototype II DMKR would appear to be EN-1655, hydromorphone, naltrexone, oxymorphone and naloxone. On the other hand, the order of affinity of some of these substrates toward the prototype III DMKR of rabbit would appear to be naloxone, oxymorphone, and naltrexone. Hydromorphone, naloxone, naltrexone, oxymorphone, and EN-1655 are probably similar as substrates for Type I DMKR's.

The substrate receptors of DMKR's do not seem to be related to CNS opiate receptors. While the antagonist naloxone and agonist oxymorphone are quite similar as substrates in the DMKR system, these compounds display major differences in CNS opiate receptor binding after treatment with the protein modifying reagent iodoacetamide (51) (Table 8).

TABLE 8

Effect of iodoacetamide on receptor binding of ^{3}H-opiate agonists and antagonists

Opiate	Stereospecific opiate binding (c.p.m) Iodoacetamide Control	Treated	% Change
Antagonists			
^{3}H-naloxone	1,040	1,092	+ 5
^{3}H-levallorphan	1,551	1,672	+ 8
Agonists			
^{3}H-oxymorphone	719	401	–44
^{3}H-levorphanol	1,288	827	–36
^{3}H-dihydromorphine	1,871	878	–53

Rat brains were homogenised in 20 volumes of standard Tris buffer and centrifuged at 50,000g for 15 min. The pellet was resuspended in 100 volumes of standard Tris buffer and equal volumes were incubated in the presence and absence of 20mM iodoacetamide for 20 min at 25° C. The homogenates were then centrifuged as before, resuspended in their original volumes and assayed with either (+)-3-hydroxy-N-allylmorphinan or (–)-3-hydroxy-N-allylmorphinan(levallorphan) at 200mM and the appropriate ^{3}H-opiate. Samples were filtered and counted. The following concentrations of ^{3}H-opiates were used: 1.7nM ^{3}H-naloxone; 2.8 nM ^{3}H-levallorphan; 3.7nM ^{3}H-oxymorphone; 4nM ^{3}H-levorphanol, and 0.7nM ^{3}H-dihydromorphine.

[Data from Wilson et al. (51).]

DMKR's and DCKR's presumably are involved in some aspect of intermediary metabolism, but their possible conspecificity with other enzymes and normal biological roles remain to be clarified. DMKR's and DCKR's nevertheless are active in xenobiosis, functioning in a capacity of detoxification as evidenced by structure-activity relationships between dihydromorphinones as well as dihydrocodeinones and their 6-keto reduction products.

Potent narcotic analgesic activity is associated with a

6-ketone moiety. Hydromorphone (7,8-dihydromorphinone) has ten times the analgetic potency of morphine in man (52), whereas the narcotic agonist activity of dihydromorphine seems to be about three times that of morphine (53,54). Dihydroisomorphine (6-*iso*-7,8-dihydromorphine or "dihydro-α-isomorphine" according to nomenclature by some authors) is weaker than dihydromorphine, being very similar to morphine in its potency (55,56). Dihydrocodeinone (3-0-methyl-dihydromorphinone) is also similar to morphine in potency (53,57), whereas dihydrocodeine seems to be at least six times less potent as an antinociceptive agent (53,58). Dihydroisocodeine appears to be closer to morphine in analgetic potency (56,59). While oxymorphone (14-hydroxydihydromorphinone) is ten times more potent than morphine in man (58) and oxycodone (14-hydroxydihydrocodeinone) appears to have about four times the antinociceptive potency of morphine as determined by the phenylquinone writhing test in mice (60), their 6-keto reduction products would be expected to have weaker agonist activity.

Potent narcotic antagonist activity is associated with appropriate N-substitution, a 6-keto group and a 14-hydroxyl moiety (61). N-allyl-7,8-dihydronormorphinone has been estimated to be about 1.3 times more potent than nalorphine (N-allylnormorphine) as a narcotic antagonist (62). As measured by the rat tailflick response, N-allyldihydronormorphinone lacked antinociceptive activity whereas nalorphine had less than 0.1 the analgesic activity of norphine (62). N-allyldihydronormorphine was found to have 0.7 the antagonist potency of nalorphine and was also weaker than nalorphine in agonist activity (62). Naloxone (N-allyl-14-hydroxydihydronormorphinone) is between ten to nineteen times more potent than nalorphine as a narcotic antagonist (63,64). While nalorphine is equianalgetic to morphine in man (65,66), naloxone at most has very minimal antinociceptive activity (63,64,67). Naltrexone (N-cyclopropylmethyl-14-hydroxydihydronormorphinone) has been determined to have up to thirty-nine times the potency of nalorphine as a narcotic antagonist and possesses very minimal agonist activity (63,64). In man naltrexone is longer acting than naloxone and is about seventeen times more potent than nalorphine as an antagonist (68). The 6-keto reduction products of naloxone and naltrexone display a substantial decrease in narcotic antagonist activity. EN-2265 (6α-naloxol) is only about twice as potent as nalorphine as an antagonist (69) and 6β-naloxol is also a weak antagonist (21,43). EN-2260 (6α-naltrexol) is similar in potency to 6β-naloxol as a narcotic antagonist and 6β-naltrexol is even weaker. Whereas 6α-naloxol has been found to possess up to about one-fifth the analgesic potency of morphine (69) and 6α-naltrexol also possesses significant analgesic activity, their 6β-hydroxy epimers are practically devoid of

antinociceptive activity (43). EN-1655 (N-cyclobutylmethyl-14-hydroxydihydronormorphinone) has about one-third the potency of naloxone as a narcotic antagonist but has antinociceptive potency in the morphine range (63,64). Nalbuphine (6α-hydroxy reduction product of EN-1655) also is very similar to morphine in analgesic potency but has only about one-fourth the potency of nalorphine as an opiate antagonist (70). Nalmexone (N-3´,3´-dimethylallyl-14-hydroxydihydronormorphinone) is a weak narcotic antagonist possessing roughly one-half the potency of nalorphine and is about one-third as active as morphine in antinociception (63,64).

Despite the low narcotic antagonist potency of 6β-naltrexol, there is now some evidence that this metabolite contributes to the relatively long duration of narcotic antagonist action of naltrexone in man (71,72). On the other hand, naloxone has been found to demonstrate narcotic agonist activity in the pigeon as determined by key pecking activity in response to stimulus presentations (73,74,75) and 6α-naloxol also possesses such activity (73). Although naloxone also shows some agonist activity in behavioral experiments in monkeys (74,75), naloxols would not be expected to be generated in sufficient quantity in such mammals to make a significant contribution toward that activity.

FURTHER STEREOSPECIFIC STUDIES IN VITRO

After the discovery of enzymes with Types III, IV, and V DMKR activity, an investigation was undertaken to obtain further information on species stereospecificity in the metabolism of dihydromorphinones and dihydrocodeinones. Since Pollock and Dear (37) obtained only 6β-naloxol when naloxone was reduced using a dialyzed salted-out rat hepatic cytosol preparation whereas Misra *et al.* (3) reported a predominance of 6α-naloxol and some 6β-naloxol produced by a more crude hepatic preparation of rat, the methodology was modified to directly employ 10 ml of hepatic supernatant prepared by ultracentrifugation at R_{ave} = 130,300 x g (R_{max} = 176,000 x g) and the endogenous NADPH generating systems were supplemented with 120 µmole glucose-6-phosphate, 7.5 µmole $NADP^+$ and 30 units crystalline glucose-6-phosphate dehydrogenase. GLC analysis was accomplished on a 1.0m x 3mm i.d. glass column packed with 3% OV-17 on 100/120 mesh using a Shimadzu Gas Chromatograph GC-5A with an FID. The column temperature was at 210°C while the injector and detector were at 290°C. Air and hydrogen flows were at 0.9 L/min and 35 ml/min respectively and N_2 flow was at 60 ml/min. GLC-MS was obtained for confirmation of metabolite identification.

A reference batch of 14-hydroxydihydroisocodeine was prepared by formamidinesulfinic acid reduction of oxycodone

with a slight modification of conditions used by Chatterjie *et al.* (43) for preparation of 6β-hydroxy reduction products of naloxone and naltrexone. A quantity of 2 mmole oxycodone·HCL was reduced in a 500 ml Ehrlenmeyer flask employing 16 mmole formamidinesulfinic acid in 130 ml aqueous solution basified to pH 12.0 with 2.22g NaOH. The reaction was allowed to proceed under a current of N_2 for ninety minutes on a hot plate at 85°C employing a magnetic stirrer. The remaining oxycodone (10%) and reduction product (90%) were then extracted into 200 ml ethyl acetate and evaporated to dryness. Analysis by GLC and confirmation by GLC-MS indicated stereospecific reduction to 14-hydroxydihydroisocodeine.

Results of stereospecific comparison of hydromorphone, naloxone, hydrocodone, and oxycodone as substrates for DMKR enzyme systems are summarized in Table 9.

In species such as pigeon, muscovy duck, and guinea pig it is possible that both dihydromorphinones and dihydrocodeinones are reduced by Type I DMKR as previously considered for the chicken DMKR system. Further studies may reveal that these species also possess Type IV DMKR (Type I DCKR). The data clearly suggests, however, that species such as rat and hamster are like rabbit in that they possess distinct enzymes with Type II and Type IV DMKR activity.

TABLE 9

Configuration of C-6 Reduction Products in vitro

	Hydromorphone	Naloxone	Hydrocodone	Oxycodone
Chicken	α	α	α	α
Pigeon	α	α	α	α
Muscovy duck	α,β	α,β	α	α
Guinea pig	α,β	α,β	α	α,β
Rabbit	β	α,β	α	α,β
Rat	β	α,β	α	α
Hamster	β	α,β	α	α

Substrates were completely metabolized by hepatic cytosol employing the supplemental NADPH generating system.

Rabbit, rat, and hamster preparations produced dihydroisomorphine rather than dihydromorphine but roughly 10% 6α-naloxol and 90% 6β-naloxol. Thus besides rabbit, rat and hamster also appear to possess Type III DMKR activity. Guinea pig like rabbit also possesses Type V DMKR (Type II DCKR) activity (Table 10).

TABLE 10

DMKR Enzyme Type	Configuration of 6-OH Product
I. Dihydromorphine: $NADP^+$ oxidoreductase (chicken, guinea pig, muscovy duck, pigeon)	α
II. Dihydroisomorphine: $NADP^+$ oxidoreductase (rabbit, rat, hamster, muscovy duck)	β
III. 14-Hydroxydihydromorphine: $NADP^+$ oxidoreductase (hamster, rabbit, rat)	α
IV. Dihydrocodeine: $NADP^+$ oxidoreductase (hamster, rabbit, rat, chicken, guinea pig, muscovy duck, pigeon)	α
V. 14-Hydroxydihydroisocodeine: $NADP^+$ oxidoreductase (rabbit, guinea pig)	β

STEREOSPECIFIC STUDIES IN VIVO

The isolation of free 6β-naltrexol from human urine (18) stimulated stereochemical studies of narcotic antagonist metabolites from laboratory animals. The *in vivo* naloxone reduction product from chicken hen, for instance, was then determined to be 6α-naloxol (19), but it was estimated that as much as 5% of the C-6 diastereomer could have been undetected (21). Malspeis *et al.* (42) reviewed and conducted some *in vivo* studies with naltrexone and determined "*that substantially greater quantities of β-naltrexol and/or its conjugates were excreted in the urine of man, monkey, guinea pig and rabbit after administration of naltrexone, whereas very small quantities were excreted by the mouse, rat and dog.*" They also detected trace amounts of 6α-naltrexol in the urine of monkey and guinea pig, the latter finding being consistent with that of Cone *et al.* (20).

In vitro studies of naloxone, naltrexone, and hydromorphone metabolism generally have correlated well with *in vivo* metabolic studies. For instance, Pollock and Dear (37) obtained a very sensitive computerized mas spectral scan of metabolites fractionated by GLC which detected no 6β-carbinol produced from naloxone by chicken cock or hen liver. On the other hand, hepatic cytosol of guinea pig liver usually produces a predominance of 6α-naloxol and 6α-naltrexol (*ca.* 95%). The stereospecific differences observed for guinea pig hepatic preparations and *in vivo* metabolism of naltrexone

remain to be reconciled but may well be resolved when the stereospecificity of guinea pig kidney DMKR's are investigated.

During preliminary *in vitro* studies by Pollock (22,23), Roerig *et al.* (76) reported dihydromorphine-3-glucuronide as an *in vivo* metabolite of dihydromorphinone in the rabbit and still suggest it is a major metabolite (36). Since rabbit *in vitro* studies by Pollock and Dear (37) yielded only the 6-*iso*-diastereomer of dihydromorphine, *in vivo* studies were undertaken to establish the C-6 stereoconfiguration of the dihydromorphinone reduction product in rabbit.

Two 4.0 kg male New Zealand white rabbits were administered 60 mg doses of hydromorphone·HCl subcutaneously twice a day for two days. Urine was collected and refrigerated at 4°C every day for seven days. The total urine from each rabbit was then centrifuged and basified to pH 10 with KOH pellets and $KHCO_3$ powder. Metabolites were extracted from basified urine into twice a volume of ethyl acetate and were concentrated by evaporation.

The residual urine was acid hydrolyzed and treated in the same manner as described for unhydrolyzed urine. Analysis by GLC and confirmation by GLC-MS revealed the biotransformation of dihydromorphinone to dihydro-6-isomorphine rather than dihydromorphine and most of the dihydroisomorphine occurred as the free metabolite. Naltrexone was reduced in a 4.0 kg male rabbit primarily to 6β-naltrexol, although about 1% 6α-naltrexol is formed and both 6α and 6β-OH reduction products were obtained from oxycodone as lagomorph urinary metabolites *in vivo*. Since Roerig *et al.* (36,76) have neither undertaken a stereochemical investigation of dihydromorphinone metabolism nor considered the findings of their own collaborators (19,21) with respect to stereospecific metabolism of the dihydromorphinone derivatives naloxone and naltrexone, we must assume their glucuronide metabolite was actually dihydroisomorphine.

From the initial human work of Cone (18) and comprehensive stereospecific studies of naltrexone reduction by Malspeis *et al.* (42), the contention by Chatterjie *et al.* (36) that the predominant 6-carbinol produced from naloxone in man is N-allyl-14-hydroxy-7,8-dihydronormorphine rather than the 6β-hydroxy diastereomer should be disregarded. The stereospecific metabolism of naltrexone and hydrocodone was investigated *in vivo* in hamsters. Three male golden Syrian hamsters (weight about 135 g each) were administered 60 mg/kg naltrexone·HCl i.p. twice a day for three days and another three were given hydrocodone·bitartrate 60 mg/kg i.p. b.i.d. for three days. Urine was collected for a week and analyzed by GLC. Free 6β-naltrexol and dihydrocodeine (6α-OH) were detected in agreement with findings from *in vitro* studies (77). Oxycodone (60 mg/kg i.p. twice a day for two days) was administered to two male Harther strain guinea pigs (460 g and 475

g) and urine was collected for a week, metabolites being analyzed by GLC. Nearly equivalent amounts of free 6α-OH and 6β-OH carbinols were detected reflecting *in vitro* Type I and II DCKR activity.

OTHER METABOLIC PATHWAYS

A recently discovered human metabolite of naltrexone is 2-hydroxy-3-0-methylnaltrexol (78,79). The aromatic hydroxylation pathway would be expected to be mediated by the microsomal phenolase complex (80) and the 3-0-methylation step by catechol-0-methyltransferase (81). Indeed, morphine, nalorphine, phenazocine and levorphanol have been demonstrated by an *in vitro* rabbit hepatic preparation to undergo aromatic hydroxylation and subsequent 0-methylation by catechol-0-methyl transferase (82). Although enantiomeric substrate stereoselectivity in the metabolism of narcotic drugs has been investigated only with respect to N-dealkylation, this type of stereospecificity would be expected to be operative for other opiate metabolic pathways as well. While enantiomeric substrate stereoselectivity should occur in the aromatic hydroxylation, 3-0-methylation and 6-keto reduction steps in the formation of 2-hydroxy-3-0-methylnaltrexol, α,β-diastereomeric product stereoselectivity also would be expected to occur in the 6-keto reduction step for which the stereochemistry of the C-6 alcohol has not yet been reported. Most likely a mixture of diastereomers is produced, but the 6β-hydroxy epimer would be expected to predominate in man.

Another type of product stereoselectivity in opiate metabolism occurs in formation of geometrical isomers, in particular *cis-trans* isomers. An example of such stereospecificity occurs in hydroxylation of the terminal methyl groups of the N-dimethylallyl substituent of pentazocine to form the *cis* and *trans* isomers 1,2,3,4,5,6-hexahydro-8-hydroxy-α,6(eq.),11(ax)-trimethyl-2,6-methano-3-benzazocine-3-*cis*-2-buten-1-ol and 1,2,3,4,5,6-hexahydro-8-hydroxy-α,6-(eq.),11(ax)-trimethyl-2,6-methano-3-benzazocine-3-*trans*-2-buten-1-ol. Mouse liver 10,000 x g supernatant produced five to ten times more *trans*-alcohol than *cis*-alcohol but rat liver formed two to three times more *cis* isomer than *trans* isomer (83). Incubation of pentazocine with rhesus monkey hepatic enzyme preparation yielded nearly equivalent amounts of *cis* and *trans* isomers. In addition some *trans*-alcohol was stereoselectively metabolized to the *trans*-carboxylic acid 1,2,3,4,5,6-hexahydro-8-hydroxy-α,6(eq.),11(ax)-trimethyl-2,6-methano-3-benzazocine-3-crotonic acid (83).

This preferential formation of *trans*-metabolites from pentazocine in rhesus monkey was also observed *in vivo* by analysis of enzymatically hydrolyzed urine (83). Both *cis*,

trans product stereoselectivity and *cis, trans* substrate stereoselectivity are also operative in the human metabolism of pentazocine. Although unaltered pentazocine, the *cis*-alcohol, and the *trans*-carboxylic acid are observed as metabolites from hydrolyzed human urine, some *trans*-alcohol would be expected as an additional metabolite (83) since it is an intermediate in formation of the *trans*-acid.

As a final example of metabolic stereospecificity of opiates, it may be pointed out that Malspeis *et al.* (42) observed that 6α-naltrexol but not 6β-naltrexol yielded an unidentified urinary metabolite after *in vivo* metabolism by guinea pigs. This represents α,β-diastereomeric substrate stereoselectivity.

CONCLUSION

Metabolic reactions are catalyzed by enzymes which tend to be asymmetrical by virtue of their numerous asymmetric centers. Biological economy thus dictates that stereospecificity will probably occur in the metabolism of opiate agonist and antagonist drugs whenever isomers are metabolized or produced as a result of metabolic reactions. Since different species may be expected to possess some differences in amino acid sequences of enzyme proteins, species differences in metabolic stereospecificity would be expected and indeed have been observed for all types of opiate metabolic stereospecificity examined.

Species variable substrate stereoselectivity of enantiomers has been demonstrated for a variety of opiates with respect to N-dealkylation. This enantiomeric stereoselectivity would be expected to occur in other opiate metabolic pathways such as glucuronidation, ethereal sulfate formation, keto reduction, O-methylation, O-dealkylation, and hydroxylation.

Species variable product stereoselectivity for geometrical isomers has been demonstrated with respect to N-alkyl hydroxylation of pentazocine and would be expected to occur in other situations where *cis-trans* isomers may be metabolically produced.

Substrate stereoselectivity of geometrical isomers has been observed in the oxidation of *cis*- and *trans*-alcohols to carboxylic acids as evidenced in the preferential metabolic formation of a *trans*-carboxylic acid after N-alkyl hydroxylation of pentazocine. Future studies may be expected to reveal instances where a species specific preference is displayed for formation of *cis*-carboxylic acids in opiate metabolism.

The most recently studied type of species variable stereospecificity in opiate metabolism is product stereoselectivity for α,β-hydroxy diastereomers as observed in the

6-keto reduction of dihydromorphinones and dihydrocodeinones. Five different types of dihydromorphinone ketone reductase (DMKR) enzyme activities have been demonstrated and other types will probably be found in the future. The stereospecific reductions of dihydromorphinones and dihydrocodeinones have not yet been shown to be reversible. If the reverse reactions were to occur, one would expect diastereomeric substrate stereoselectivity to be demonstrated. For example, the oxidation of dihydromorphine would not be catalyzed by Type II DMKR's and the oxidation of dihydroisomorphine would not be mediated by Type I DMKR's.

It is noteworthy that muzzling of the phenolic 3-hydroxyl moiety of dihydromorphinones as occurs in dihydrocodeinones not only produces a diminution of analgesic potency but causes a significant change in stereospecific metabolism. Although there are significant differences in stereospecificity of opiates toward enzyme active sites and toward analgesic CNS receptor sites, stereochemical factors in the metabolism of narcotic agonist and antagonist drugs undoubtedly have a profound influence on pharmacological activity and certainly warrant further investigation.

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REFERENCES

1. Way, E.L. and Adler, T.K.: The pharmacologic implications of the fate of morphine and its surrogates. *Pharmac. Rev. 12*:383-446 (1960).
2. Way, E.L.: Distribution and metabolism of morphine and its surrogates. *Res. Publ. Assoc. Res. Nerv. Ment. Dis. 46*:13-31 (1968).
3. Misra, A.L., Pontani, R.B., Vadlamani, N.L. and Mule, S. J.: Physiological disposition and biotransformation of [allyl-1',3'-^{14}C] naloxone in the rat and some comparative observations on nalorphine. *J. Pharmacol. exp. Ther. 196(2)*:257-268 (1976).
4. Weinstein, S.H., Pfeffer, M., Schor, J.M., Indindoli, L., and Mintz, M.: Metabolites of naloxone in human urine.

J. Pharm. Sci. 60:1567-1568 (1971).
5. Axelrod, J.: The enzymatic N-demethylation of narcotic drugs. *J. Pharmacol. exp. Ther. 117*:322-330 (1956).
6. Axelrod, J. and Cochin, J.: Inhibitory action of N-allylnormorphine on enzymatic demethylation of narcotic drugs. *Fed. Proc. 15*:395 (1956).
7. Axelrod, J.: Possible mechanism of tolerance to narcotic drugs. *Science 124*:263-264 (1956).
8. Elison, C., Elliott, H.W., Look, M. and Rapoport, H.: Some aspects of the fate and relationship of the N-methyl group of morphine to its pharmacological activity. *J. Med. Chem. 6*:237-246 (1963).
9. Beckett, A.H., Casy, A.F. and Harper, N.J.: Analgesics and their antagonists: Some steric and chemical considerations: Part II. The influence of the basic group on the biological response. *J. Pharm. Pharmac. 8*:874-883 (1956).
10. Axelrod, J. and Cochin, J.: The inhibitory action of nalorphine on the enzymatic N-demethylation of narcotic drugs. *J. Pharmacol. exp. Ther. 121*:107-112 (1957).
11. Milthers, K.: N-dealkylation of morphine and nalorphine in the brain of living rats. *Nature 195*:607 (1962).
12. Takemori, A.E. and Mannering, G.J.: Metabolic N- and O-demethylation of morphine- and morphinan-type analgesics. *J. Pharmacol. exp. Ther. 123*:171-179 (1958).
13. Jenner, P. and Testa, B.: The influence of stereochemical factors on drug disposition. *Drug Metab. Rev. 2(2)*: 17-184 (1973).
14. Fujimoto, J.M.: Isolation of two different glucuronide metabolites of naloxone from the urine of rabbit and chicken. *J. Pharmacol. exp. Ther. 168(1)*:180-186 (1969).
15. Fujimoto, J.M.: Isolation of naloxone-3-glucuronide from human urine. *Proc. Soc. Exp. Biol. Med. 133(1)*:317-319 (1970).
16. Weinstein, S.H., Pfeffer, M. and Schor, J.M.: Metabolism and pharmacokinetics of naloxone, *Narcotic Antagonists. Advances in Biochemical Psychopharmacology 8*. Raven Press, New York, p. 525-535 (1974).
17. Gates, M. and Montzka, T.A.: Some potent morphine antagonists possessing high analgesic activity. *J. Med. Chem. 7(2)*:127-131 (1964).
18. Cone, E.J.: Human metabolite of naltrexone (N-cyclopropylmethylnoroxymorphone) with a novel C-6 isomorphine configuration. *Tetrahedron Lett.* No. 28:2607-2610 (1973).
19. Chatterjie, N., Fujimoto, J.M., Inturrisi, C.E., Roerig, S., Wang, R.H., Bowen, D.V., Field, F.H. and Clarke, D.D.: Isolation and stereochemical identification of a metabolite of naltrexone from human urine. *Drug Metab. Dispos. 2(5)*:401-405 (1974).

20. Cone, E.J., Gorodetzky, C.W. and Yeh, S.Y.: Biosynthesis, isolation and identification of 6β-hydroxynaltrexone, a major human metabolite of naltrexone. *J. Pharm. Sci. 64(4)*:618-621 (1975).
21. Fujimoto, J.M., Roerig, S., Wang, R.I.H., Chatterjie, N. and Inturrisi, C.E.: Narcotic antagonist activity of several metabolites of naloxone and naltrexone tested in morphine dependent mice. *Proc. Soc. Exp. Biol. Med. 148*:443-448 (1975).
22. Pollock, S.H.: Metabolic studies on some important narcotic analgesic agonist and antagonist drugs: A partial characterization of dihydromorphinone ketone reductases. Part I of Pharmacology Master of Science thesis defended at the Medical College of Wisconsin in Milwaukee, May, 1974, and submitted in final form, April, 1975.
23. Pollock, S.H.: Dihydromorphinone ketone reductases. *Life Sciences 17*:465-475 (1975).
24. Zannoni, V.G. and Weber, W.W.: Isolation and properties of aromatic α-keto acid reductase. *J. Biol. Chem. 241(6)*:1340-1349 (1966).
25. Meister, A.: Reduction of α,γ-diketo and α-keto acids catalyzed by muscle preparations and by crystalline lactic dehydrogenase. *J. Biol. Chem. 184*:117-129 (1950).
26. Winer, A.D.: A note on the substrate specificity of horse liver alcohol dehydrogenase. *Acta Chem. Scand. 12(8)*:1695-1696 (1958).
27. Merritt, D. and Tomkins, G.M.: Reversible oxidation of cyclic secondary alcohols by liver alcohol dehydrogenase. *J. Biol. Chem. 234(10)*:2778-2782 (1969).
28. Graves, J.M.H., Clark, A. and Ringold, H.: Stereochemical aspects of the substrate specificity of horse liver alcohol dehydrogenase. *Biochemistry 4(12)*:2655-2671 (1965).
29. Elliot, T.H., Jacob, E. and Tao, R.C.C.: The *in vitro* and *in vivo* metabolism of optically active methylcyclohexanols and methylcyclohexanones. *J. Pharm. Pharmac. 21*:561-572 (1969).
30. Dutler, H., Coon, M.J., Kull, A., Vogel, H., Waldvogel, G. and Prelog, V.: Fatty acid synthetase from pig liver 1. Isolation of the enzyme complex and characterization of the component with oxidoreducatse activity for alicyclic ketones. *Eur. J. Biochem. 22*:203-212 (1971).
31. Dutler, H., Kull, A. and Mislin, R.: Fatty acid synthetase from pig liver 2. Characterization of the enzyme complex with oxidoreducatse activity for alicyclic ketones as a fatty acid synthetase. *Eur. J. Biochem. 22*: 213-217 (1971).
32. Yun, S.L. and Hsu, R.Y.: Fatty acid synthetase of

chicken liver. Reversible dissociation into two non-identical subcomplexes of similar size. *J. Biol. Chem. 247(9)*:2689-2698 (1972).

33. Culp, H.W. and McMahon, R.E.: Reductase for aromatic aldehydes and ketones. *J. Biol. Chem. 243(4)*:848-852 (1968).
34. Fraser, J.M., Peters, M.A. and Hardinge, M.G.: Purification and some characteristics of an α,β-unsaturated ketone reductase from dog erythrocytes and human liver. *Mol. Pharmacol. 3(3)*:233-247 (1967).
35. Sokal, R.R. and Rohlf, James F.: *Biometry*. W.H. Freeman & Co., San Francisco (1969).
36. Roerig, S., Fujimoto, J.M., Wang, R.I.H., and Lange, D.: Preliminary characterization of enzymes for reduction of naloxone and naltrexone in rabbit and chicken liver. *Drug Metab. Dispos. 4(1)*:53-58 (1976).
37. Pollock, S.H. and Dear, L.L.: Unpublished findings, some of which were presented to the American Society for Pharmacology and Experimental Therapeutics in Davis, California, August 25, 1975; abstracted as "Stereospecific studies of hepatic dihydromorphinone ketone reductases using naloxone as a model substrate. *Pharmacologist 17(2)*:239 (1975).
38. Pollock, S.H.: Unpublished findings and observations.
39. Rendina, G.: *Experimental Methods in Modern Biochemistry*. W.B. Saunders, Philadelphia (1971).
40. Hays, W.L.: *Statistics*. Holt, Rinehart, and Winston, New York (1963).
41. Hahn, E.F. and Fishman, J.: Narcotic antagonists. V. Stereochemisty of reactions at C-6 in 14-hydroxynoroxymorphone derivatives. *J. Org. Chem. 40(1)*:31-34 (1975).
42. Malspeis, L., Bathala, M.D., Ludden, T.M., Bhat, H.B., Frank, S.G., Sokoluski, T.D., Morrison, B.E. and Reuning, R.H.: Metabolic reduction of naltrexone I. Synthesis, separation and characterization of naloxone and naltrexone reduction products and qualitative assay of urine and bile following administration of naltrexone, α-naltrexol, or β-naltrexol. *Res. Commun. Chem. Pathol. Pharmacol. 12(1)*:43-65 (1975).
43. Chatterjie, N., Inturrisi, C.E., Dayton, H.B. and Blumberg, H.: Stereospecific synthesis of the 6β-hydroxy metabolites of naltrexone and naloxone. *J. Med. Chem. 18(5)*:490-492 (1975).
44. Barman, T.E.: *Enzyme Handbook* (Volumes I and II). Springer-Verlag, Berlin (1969) and Supplement I (1974).
45. Boyer, P.D. (editor): *The Enzymes*. Academic Press, New York (1974).
46. Bruice, T.C. and Benkovic, S.J.: *Bioorganic Mechanisms* (Volumes I and II). W.A. Benjamin, New York (1966).

47. King, T.E., Mason, H.S. and Morrison, M. (editors): *Oxidases and Related Redox Systems* (Volumes I and II). University Park Press, Baltimore (1973).
48. Whitehouse, M.W.: *Lipid Pharmacology*. Academic Press, New York, p. 185-236 (1964).
49. Dorfman, R.I. and Unger, F.: *Metabolism of Steroid Hormones*. Academic Press, New York (1965).
50. Ober, K.F. and Fujimoto, J.M.: Isolation of naloxone-3-ethereal sulfate from urine of the cat. *Proc. Soc. Exp. Biol. Med. 139(3)*:1068-1070 (1972).
51. Wilson, H.A., Pasternak, G.W. and Snyder, S.H.: Differentation of opiate agonist and antagonist receptor binding by protein modifying reagents. *Nature 253*:448-450 (1975).
52. Mahler, D.L. and Forrest, W.H. Jr.: Relative analgesic potencies of morphine and hydromorphone in postoperative pain. *Anesthesiology 42(5)*:602-607 (1975).
53. Eddy, N.B. and Reid, J.G.: Studies of morphine, codeine and their derivatives VII. Dihydromorphine (Paramorphan), dihydromorphinone (Dilaudid), and dihydrocodeinone (Dicodide). *J. Pharmacol. exp. Ther. 52*:468-493 (1934).
54. Himmelsbach, C.A.: Studies of certain addiction characteristics of (a) dihydromorphine ("Paramorphan") (b) dihydrodesoxymorphine-D ("Desomorphine") (c) dihydrodesoxycodeine-D ("desocodeine") and (d) methyldihydromorphinone ("metopon"). *J. Pharmacol. exp. Ther. 67*:239-249 (1939).
55. Wright, C.I. and Barbour, F.A.: The respiratory effects of morphine, codeine, and related substances V. The effect of α, β, γ, dihydro-α-,dihydro-β-, and dihydro-γ-isomorphine on the respiration of the rabbit. *J. Pharmacol. exp. Ther. 56*:39-49 (1936).
56. Eddy, N.B.: Studies of morphine, codeine and their derivatives XII. The isomers of morphine and dihydromorphine. *J. Pharmacol. exp. Ther. 56*:421-431 (1936).
57. Wright, C.I. and Barbour, F.A.: The respiratory effects of morphine, codeine and related substances. III. The effect of morphine, dihydromorphine, dihydromorphinone (Dilaudid) and dihydrocodeine (Dicodid) on the respiratory activity of the rabbit. *J. Pharmacol. exp. Ther. 53*:34-45 (1935).
58. Lasagna, L.: The clinical evaluation of morphine and its substitutes as analgesics. *Pharmac. Rev. 16(1)*:47-83 (1964).
59. Eddy, N.B.: Studies of morphine, codeine and their derivatives IV. Hydrogenated codeine isomers. *J. Pharmacol. exp. Ther. 51*:35-44 (1934).
60. Vernier, V.: Personal communication to S. Pollock of data obtained by H.B. Dayton and V. Vernier at Endo

Laboratories.
61. Robinson, F.M.: Chemistry of narcotic antagonists of the nalorphine type, *Narcotic Antagonists. Advances in Biochemical Psychopharmacology 8.* Raven Press, New York, p. 21-31 (1974).
62. Winter, C.A., Orahovats, P.D. and Lehman, E.G.: Analgesic activity and morphine antagonism of compounds related to nalorphine. *Arch. int. Pharmacodyn. 110(2-3)*:186-202 (1957).
63. Blumberg, H. and Dayton, H.B.: Naloxone and related compounds, *Agonist and Antagonist Actions of Narcotic Analgesic Drugs.* University Park Press, Baltimore, p. 110-119 (1973).
64. Blumberg, H. and Dayton, H.B.: Naloxone, naltrexone, and related noroxymorphones, *Narcotic Antagonists. Advances in Biochemical Psychopharmacology 8.* Raven Press, New York, p. 33-43 (1974).
65. Lasagna, L. and Beecher, H.K.: The analgesic effectiveness of nalorphine and nalorphine-morphine combinations in man. *J. Pharmacol. exp. Ther. 112*:356-363 (1954).
66. Keats, A.S. and Telford, J.: Nalorphine, a potent analgesic in man. *J. Pharmacol. exp. Ther. 117*:190-196 (1956).
67. Lasagna, L.: Drug interaction in the field of analgesic drugs. *Proc. R. Soc. Med. 58(2)*:978-983 (1965).
68. Martin, W.R., Jasinski, D.R. and Mansky, P.A.: Naltrexone, an antagonist for the treatment of heroin dependence. *Arch. gen. Psychiat. 28*:784-791 (1973).
69. Dayton, H.B. and Blumberg, H.: A comparison of the narcotic antagonist naloxone and its 6-hydroxy analog EN-2265. *Fed. Proc. 28(1)*:736 (1969).
70. Jasinski, D.R. and Mansky, P.A.: Evaluation of nalbuphine for abuse potential. *Clin. Pharmacol. Ther. 13(1)*: 78-90 (1972).
71. Cone, E.J., Gorodetzky, C.W. and Yeh, S.Y.: The urinary excretion profile of naltrexone and metabolites in man. *Drug Metab. Dispos. 2(6)*:506-512 (1974).
72. Verebely, K., Mule, S.J. and Jukofsky, D.: A gas-liquid chromatographic method for the determination of naltrexone and beta-naltrexol in human urine. *J. Chromatogr. 111*:141-148 (1975).
73. McMillan, D.E., Wolf, P.S. and Carchman, R.A.: Antagonism of the behavioral effects of morphine and methadone by narcotic antagonists in the pigeon. *J. Pharmacol. exp. Ther. 175*:443-457 (1970).
74. Downs, D.A. and Woods, J.H.: Morphine, pentazocine and naloxone effects on responding under a multiple schedule of reinforcement in rhesus monkeys and pigeons. *J. Pharmacol. exp. Ther. 196(2)*:298-306 (1976).

75. Goldberg, S.R., Morse, W.H. and Goldberg, D.M.: Some behavioral effects of morphine, naloxone, and nalorphine in the squirrel monkey and the pigeon. *J. Pharmacol. exp. Ther. 196(3)*:625-636 (1976).
76. Roerig, S., Fujimoto, J.M. and Wang, R.I.H.: Isolation of hydromorphone and dihydromorphine glucuronides from urine of the rabbit after hydromorphone administration. *Proc. Soc. Exp. Biol. Med. 143*:230-233 (1973).
77. Pollock, S.H., Blum, K., Dear, L.L., Futterman, S., Wallace, J.E. and Schwertner, H.A.: A stereospecific comparison of the enzymatic 6-keto reduction of hydromorphone, naloxone, hydrocodone and oxycodone. *Pharmacologist 18(2)*:374 (1976).
78. Verebely, K., Chedekel, M.A., Mule, S.J. and Rosenthal, D.: Isolation and identification of a new metabolite of naltrexone in human blood and urine. *Res. Commun. Chem. Pathol. Pharmacol. 12(1)*:67-84 (1975).
79. Cone, E.J. and Gorodetzky, C.W.: Metabolism of naltrexone and naloxone. *NAS-NRC Committee on Problems of Drug Dependence*, p. 338-347, May (1975).
80. Mason, H.S.: Comparative biochemistry of the phenolase complex. *Advances in Enzymology 16*:105-184 (1955).
81. Axelrod, J. and Tomchick, R.: Enzymatic O-methylation of epinephrine and other catechols. *J. Biol. Chem. 233(3)*: 702-705 (1958).
82. Daly, J., Inscoe, J.K. and Axelrod, J.: The formation of O-methylated catechols by microsomal hydroxylation of phenols and subsequent enzymatic catechol O-methylation. Substrate specificity. *J. Med. Chem. 8*:153-157 (1965).
83. Pittman, K.A., Rosi, D., Cherniak, R., Merola, A.J. and Conway, W.D.: Metabolism *in vitro* and *in vivo* of pentazocine. *Biochem. Pharmacol 18(2)*:1673-1678 (1969).